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ASCORBIC ACID DEFICIENCY AND LIVER DISEASE:

EFFECTS ON ETHANOL OXIDIZING ENZYMES

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c

Thesis submitted for the degree of

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ABBREVIATIONS

ADH	Alcohol dehydrogenase
AA	L-Ascorbic acid
DPH	Diphenylhydantoin
GLC	Gas liquid chromatography
i.p.	intraperitoneal
LAA	Leucocyte ascorbic acid
LDH	Lactate dehydrogenase
MEOS	Microsomal ethanol oxidizing system
NAD ⁺	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP ⁺	Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
t _{1/2}	Serum half life
TCA	Tricarboxylic acid
SD	Standard deviation
SKF 525-A	2-diethylaminoethyl-2,2-diphenyl-valerate
β60	mg ethanol cleared/100ml blood/hour

SUMMARY

1. (a) A significant decrease in the extent of the redox state shift after ethanol administration was obtained in chronic ethanol treated normal and ascorbic acid deficient guinea pigs, compared with their respective controls.
(b) This was not due to differences in blood ethanol concentration between acute and chronic ethanol treated animals, as the theoretical zero time blood ethanol concentration was the same in all the groups of animals.
(c) The decrease in the redox state shift after chronic ethanol administration may represent metabolic adaptation to ethanol at the mitochondrial level. As this was achieved after a relatively short period of exposure to ethanol, it is likely that it can occur in man after exposure to comparable quantities of ethanol.
(d) Although the redox state is important in the regulation of ethanol elimination in vivo, the approximately 10 per cent decrease in its shift, after ethanol administration to chronic ethanol treated animals, had no effect on their rate of ethanol elimination.
(e) There was no statistical difference between the redox states of ascorbic acid deficient groups and their corresponding normal controls.
2. (a) Microsomal protein content and aniline hydroxylase and NADPH oxidase activities, were significantly reduced in ascorbic acid deficient guinea pigs, although catalase and MEOS activities were unchanged. Cytochrome P-450 content was also significantly decreased, but no change was observed in cytochrome b_5 content.
(b) Although the half life of diphenylhydantoin was increased by 36 per cent in the ascorbic acid deficient guinea pig, no change in the rate of ethanol clearance from the blood was found.

- (c) Chronic ethanol treatment did not induce microsomal enzyme activities or increase cytochrome P-450 content.
- (d) Phenobarbitone pretreatment of guinea pigs resulted in increases in liver/body wt, activity of aniline hydroxylase, cytochrome P-450 content and microsomal protein, although this had no effect on the rate of ethanol clearance from the blood.
- (e) As MEOS activity was not dependent on cytochrome P-450 content, and did not parallel changes in the activity of aniline hydroxylase, it does not have properties associated with established microsomal drug metabolizing enzymes.
3. (a) A significant correlation coefficient ($r = 0.88$, $P < 0.001$) was obtained when hepatic alcohol dehydrogenase activities of 12 patients with non-alcoholic liver disease were plotted against their corresponding leucocyte ascorbic acid content.
- (b) The rate of clearance of ethanol from the blood of 11 healthy male volunteers was significantly increased after oral ascorbic acid supplementation.
- (c) Hepatic alcohol dehydrogenase activity and leucocyte ascorbic acid content were measured in 35 patients with liver disease and in 10 control subjects with duodenal ulcer. The patients with liver disease were divided into non drinkers, moderate drinkers and alcoholic/heavy drinkers.
- (d) There was no significant difference in hepatic alcohol dehydrogenase activity between the groups with liver disease, but they had less than half the hepatic alcohol dehydrogenase activity of the control subjects.
- (e) A correlation coefficient of $r = 0.77$ ($P < 0.001$) was obtained when the hepatic alcohol dehydrogenase activity of each patient with liver disease was plotted against its corresponding leucocyte ascorbic acid content. An insignificant correlation coefficient

$r = 0.332$ was found in the control subjects, who had no evidence of liver disease.

(f) When this was repeated with the liver disease patients divided according to their previous alcohol intake, significant correlation coefficients of $r = 0.873$ ($P < 0.001$) for the non drinkers, $r = 0.739$ ($P < 0.02$) for the moderate drinkers, and $r = 0.702$ ($P < 0.005$) for the alcoholic/heavy drinkers were obtained.

(g) The addition of ascorbic acid in vitro (0.5 mM to 10 mM) had no effect on the activity of alcohol dehydrogenase.

(h) The relation between hepatic alcohol dehydrogenase activity and leucocyte ascorbic acid content is probably a consequence of liver disease, as opposed to any specific effect of ascorbic acid status on alcohol dehydrogenase activity.

4. (a) A 4% incidence of the atypical form of human alcohol dehydrogenase was found in a Scottish population. This exhibited a bimodal distribution.

5. (a) The activity of the NADPH dependent ethanol oxidizing system was increased in patients with liver disease. This was significant whether activities were expressed per mg protein or per g wet wt liver.

(b) It is suggested that the NADPH dependent ethanol oxidizing system maintains normal rates of ethanol metabolism in patients with liver disease. The greater activity of the NADPH dependent system in patients with liver disease may compensate for the low alcohol dehydrogenase activities found in these patients.

..... INTRODUCTION

Historical

The use by man of alcoholic drinks dates back to the very beginnings of human civilisation. In all the ancient civilisations of which records remain, their wealth was measured by production of corn, wine and oil. The origin of these necessities being ascribed to the gods.

The manufacture of wine has developed from the distribution of the grape vine and is characteristic of a settled civilisation. The vine does not bear fruit till three years after planting, whereas a crop of corn can be raised within a year. The possession of a vineyard thus becomes synonymous with a settled existence, and it seems probable that the invention of wine is synchronous with the birth of civilisation.

General

The metabolic effects of ethanol depend on the quantities consumed. Moderate quantities are relatively innocuous, and ethanol acts as a nutrient which can supply a large part of the caloric requirements of the organism. The consumption of ethanol, in quantities that are in excess of the upper limits of the mechanisms responsible for its metabolic elimination, leads to serious toxic effects and metabolic derangements.

The metabolism of ethanol can interfere with that of various foodstuffs and endogenous substrates, as they all share common metabolic pathways. Ethanol per se may also produce metabolic alterations by exerting a direct toxic effect on specific organs.

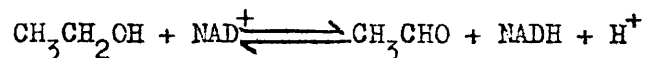
Site of Metabolism of Ethanol

Early in the 20th century, Batteli and Stern¹ demonstrated that human liver was capable of oxidizing ethanol. The central role of the liver in the oxidation of ethanol was established using perfused liver, which was shown to remove ethanol from the perfusion fluid²; and by hepatectomy, which caused ethanol oxidation in dogs to decrease to very low levels³. Later work by Tygstrup et al⁴ in man, and Gordon⁵ in other species, has established that 70 - 95% of ethanol metabolism is accounted for by oxidation in the liver.

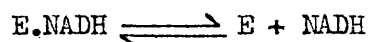
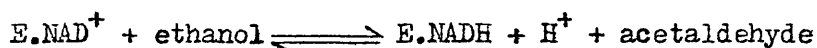
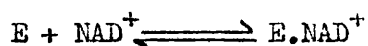
Enzymology of Ethanol Metabolism

In the liver there are three enzyme systems capable of oxidizing ethanol to acetaldehyde. These are alcohol dehydrogenase (ADH), catalase and the microsomal ethanol oxidizing system (MEOS).

(i) Alcohol Dehydrogenase (alcohol - NAD oxidoreductase EC IIII) is the initial and rate limiting enzyme in the major pathway of ethanol metabolism⁶. It is a cytosol enzyme and catalyzes the following reaction:



Bäcklin⁷ found the equilibrium constant of this reaction to be 8×10^{-12} at 20°C and ionic strength 0.1. The position of this equilibrium is very unfavourable for the oxidation of ethanol, as the change in free energy at physiological pH (ΔG , pH 7.4) is +4.9K cal/mole. Fortunately the oxidation of acetaldehyde to acetate has a very favourable change in free energy (ΔG about -13K cal/mole) which furnishes the free energy needed for the dehydrogenation of ethanol. As acetaldehyde is oxidized to acetate, more ethanol can be oxidized to acetaldehyde and thus the equilibrium of the ADH reaction can be maintained. Theorell and Chance⁸ have proposed the following reaction mechanism for ADH:



Experiments with purified ADH have shown that the rate limiting step in this reaction is the dissociation of the E.NADH complex.

Horse liver ADH, the most extensively studied of the ADH enzymes, has a molecular weight of 84000 with four atoms of zinc present in each molecule⁹. Removal of two zinc atoms by dialysis, or addition of zinc complexing agents such as EDTA, abolishes enzyme activity⁹. Human liver

ADH resembles horse liver ADH in many physicochemical and kinetic properties. Its molecular weight is 87000¹⁰ and it contains two atoms of zinc per molecule¹¹. Harris¹² has proposed that the active enzyme consists of two similar polypeptide chains, each containing a reactive -SH group in a cysteine residue. Theorell and Bonnichsen¹³ have shown that the -SH group, together with zinc and other amino acid side chains, is involved in binding the substrate and coenzyme to each of the active centres within the quaternary structure of the active dimer. Human ADH differs in substrate specificity from horse liver ADH; it can oxidize methanol, ethylene glycol¹⁰, isopropyl alcohol, chloral hydrate and other pharmacologically and toxicologically important compounds at relatively high rates¹¹.

ADH has been shown to exist in multimolecular forms; the number of ADH active bands which can be separated in liver homogenates by gel electrophoresis depends on the species¹⁴. This heterogeneity of ADH has been investigated predominantly in horse liver, although at least seven isoenzymes can be distinguished by electrophoretic and chromatographic methods in human liver¹⁵. Schenker et al¹⁶ have purified, isolated and determined the subunit composition of six of these isoenzymes from human liver. They have suggested that the isoenzymes are formed by combinations of three subunits designated A, B and B¹ to give six dimers AA, AB, BB, BB¹, B¹B¹ and AB¹. Subunit B has been shown by Berger et al¹⁷ to exist in two forms termed B₁ and B₂. These subunits differ with respect to one amino acid residue, which is located in a region of the subunit which corresponds to the coenzyme binding site of horse ADH. B₂ is considered the subunit responsible for the atypical catalytic properties discussed below.

Human liver ADH exhibits a polymorphism in addition to its heterogeneity¹⁸;

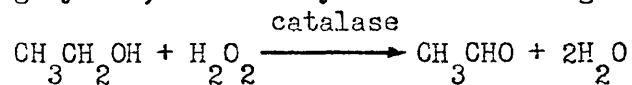
this polymorphism is revealed by a bimodal distribution of the enzyme in a population, when a screening test is used to differentiate between the normal and atypical enzyme¹⁹. The normal enzyme has a pH optimum of 10.8 while the atypical enzyme has a pH optimum of 8.5.

Development of liver ADH in the foetus and neonate has been described for the human by Pikkarainen and R  ih  ²⁰, and for the rat and guinea pig by Raiha et al²¹. Adult activities were found at 5 years of age in the human, 18 days after birth in the rat and 6 - 8 days in the guinea pig. In the human there are kinetic differences between adult ADH and that of the foetus and infant²⁰.

Since other alternative pathways for ethanol metabolism lead to the formation of the same product, acetaldehyde, the proof of ADH participation in vivo is indirect. One type of indirect evidence is the correlation between in vitro and in vivo effects of known inhibitors of ADH. Pyrazole given to rats in a dose of approximately 4mmoles/Kg shortly before removal of the liver, inhibits ADH activity completely; the same dose reduces the in vivo rate of ethanol metabolism by 80 - 100%²². The similarity of the kinetics of ADH activity in vitro, and of ethanol disappearance in vivo also furnishes indirect evidence for an in vivo role for ADH in ethanol metabolism. The disappearance of ethanol from the blood or whole body continues at a constant rate in most species until the concentration in the body water has fallen to 2 - 5 mM, depending on the species²³. Calculation of the apparent Km from the non-linear portions of the blood alcohol curves below these concentrations, yield values which agree reasonably closely with those obtained in vitro with ADH preparations from the same species²³. The Km values for the other possible pathways of ethanol metabolism are much higher²⁴.

The physiological role of ADH is to interconvert various endogenous alcohols and aldehydes. Small amounts of endogenous ethanol have been detected in the blood, tissues, and urine of subjects who have not consumed alcohol. Ethanol levels in the portal vein have been shown to exceed those in the hepatic artery, and oral administration of antibiotics²⁵ can reduce these levels. This suggests that hepatic ADH functions as a detoxification mechanism for low levels of ethanol formed in the intestinal tract.

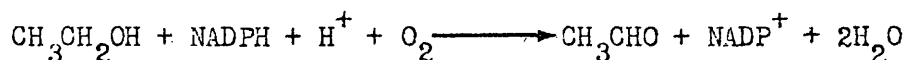
(ii) Catalase.²⁶ Keilin and Hartree showed that catalase, in the presence of a H_2O_2 generating system, can catalyze the following reaction:



In this reaction catalase is acting as a peroxidase as ethanol is the hydrogen donor. The above reaction has been demonstrated in rat liver homogenates, by Lundquist et al.²⁷ Large amounts of catalase are found in the body, and as there are also various peroxide generating systems present, it is possible that some oxidation of ethanol could occur by this route in vivo. Smith²⁸ using the catalase inhibitor 3-amino-1,2,4-triazole obtained 90% inhibition of catalase activity in vivo, although the rate of oxidation of ethanol both in vivo and in vitro was unchanged. Under normal in vivo conditions the rate of H_2O_2 formation was found to be too rate limiting to permit even a small fraction of ethanol metabolism to take place via peroxidation²⁹, therefore a system which can produce H_2O_2 and can be enhanced with chronic ethanol treatment is required. Such a system has been described by Carter and Isselbacher³⁰, who demonstrated an increase in microsomal NADPH oxidase activity in rats that were chronically exposed to ethanol. This system could therefore lead to an increased metabolism of ethanol by catalase, without any increase in catalase activity,

as most investigators have failed to show an increase in catalase activity after chronic ethanol treatment³¹. Aebi³² has shown that the production of H_2O_2 is in fact the rate limiting step in the catalase reaction. Although an increase in the activity of NADPH oxidase has been shown,³¹ Videla et al³¹ have calculated that the resulting increased production of H_2O_2 , and subsequent peroxidation of ethanol by catalase, could only account for 10 - 20% of the increased rate of ethanol metabolism in chronic ethanol treated rats. The role of catalase in NADPH dependent ethanol oxidation is discussed below.

(iii) Microsomal Ethanol Oxidizing System. Orme-Johnson and Ziegler³³ demonstrated that the mixed function oxidase system in mammalian liver microsomes, which plays a major role in the metabolism of many drugs, could also catalyze the NADPH and O_2 dependent oxidation of ethanol and methanol to their corresponding aldehydes, in vitro, by the following reaction:



As cytochrome P-450 is involved in microsomal hydroxylase reactions, CO would be expected to inhibit this reaction by binding to cytochrome P-450. This was not found by Orme-Johnson and Ziegler, although they demonstrated inhibition of the reaction with azide and cyanide. In a similar system, termed the microsomal ethanol oxidizing system (MEOS),³⁴ Lieber and De Carli³⁴ demonstrated ethanol oxidation to acetaldehyde. This system differed from the one outlined above by being sensitive to CO inhibition, and oxidizing ethanol at a higher rate than methanol. Other workers have shown that a H_2O_2 generating system can be substituted for NADPH or a NADPH generating system, although direct addition of H_2O_2 ³⁵ was ineffective. MEOS has been widely studied because of its possible

involvement in a common pathway for ethanol and drug metabolism, although a controversy exists as to its true nature. Lieber and De Carli support the concept that MEOS is a true microsomal oxidase similar to the microsomal drug oxidase system; whereas other workers consider MEOS activity to be a product of microsomal NADPH oxidase activity, which generates H_2O_2 , and catalase activity, which utilizes this generated H_2O_2 to peroxidate ethanol to acetaldehyde.

Lieber and De Carli have utilized the following observations to support their concept of MEOS. MEOS, like other microsomal drug metabolizing enzymes, requires NADPH and oxygen³⁴ and is not inhibited by pyrazole at concentrations which inhibit ADH³⁴. Thus MEOS activity is considered to be separate from ADH activity, because of this difference in pyrazole inhibition. Drugs which are oxidized by the microsomal drug metabolizing system bind to microsomes and produce spectral changes of two distinct types (I or II)³⁶ (Fig 1). As the addition of ethanol to microsomes produces a modified type II binding spectrum³⁷, this suggests that ethanol can interact with microsomes in a similar manner to drugs. Phenobarbitone pretreatment, which induces microsomal enzyme activities, also increases the activity of MEOS³⁸. Lieber and De Carli³⁹ showed that the in vivo administration of pyrazole to rats had no effect on MEOS or NADPH oxidase activity, but inhibited catalase activity by 90%. They therefore concluded that catalase was not required for the function of MEOS. The differentiation between catalase and MEOS activity was also demonstrated by the addition in vitro of azide, a powerful catalase inhibitor,⁴⁰ to microsomes of pyrazole pretreated rats. Lieber and De Carli⁴⁰ obtained an approximately 95% inhibition of H_2O_2 dependent microsomal ethanol oxidation using these inhibitors, while NADPH dependent microsomal ethanol

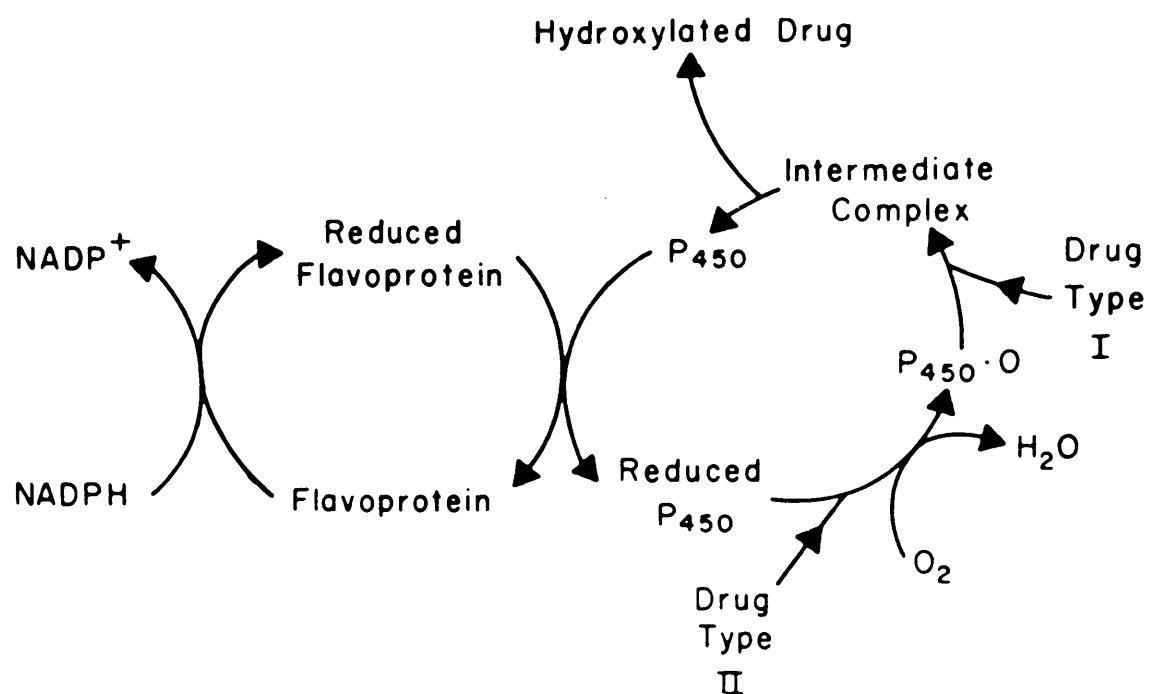


FIG. I

Type I and Type II substrates metabolized by
 cytochrome P-450 dependent drug metabolizing system.

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oxidation was decreased by only 33%.

The concept of MEOS activity being a product of NADPH oxidase and catalase activity is supported by many workers, who base this alternative system on the following observations. Similarities between MEOS, and the NADPH oxidase and catalase system, with respect to requirement for NADPH and oxygen have been found by Thurman et al⁴¹. These authors showed that both systems had identical oxygen concentrations for half maximal rates, and both systems were sensitive to CO inhibition. They also demonstrated that microsomal acetaldehyde production, in the presence of either an NADPH or H_2O_2 generating system, exhibited identical characteristics with respect to ethanol concentration for half maximal rates (12mM). Formate, a peroxidatic substrate, was shown to competitively inhibit acetaldehyde production in both generating systems; and catalase inhibitors such as azide also inhibited acetaldehyde production in both types of generating system.

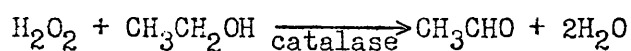
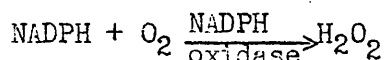
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Carter and Isselbacher have also added to the evidence for the involvement of catalase in MEOS activity. They noted that the combined use of azide and pyrazole, inhibited both catalase and the system used to generate H_2O_2 , and this combination is therefore unsuitable for use in differentiating between H_2O_2 and NADPH dependent ethanol oxidation. Instead they used catalase inhibitors such as cyanide in vitro, and aminotriazole in vivo, and demonstrated that NADPH dependent ethanol oxidation was inhibited.

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Khanna et al have also concluded that microsomal oxidation of ethanol depends on catalase like activity, again basing this on observations that catalase inhibitors markedly reduced NADPH dependent ethanol oxidation. They also showed that H_2O_2 and barium peroxide could

both replace NADPH in supporting ethanol oxidation.

Lastly, the type II binding spectrum of ethanol with microsomes, is considered by Imai and Sato⁴⁴ to be a direct action of ethanol on microsomal membranes, rather than a true substrate binding effect.

The above observations, although depending on the specificity of enzyme inhibitors, suggest that catalase is involved in NADPH dependent ethanol oxidation, and Carter and Isselbacher⁴² have proposed the following mechanism:



NADPH oxidase produces H_2O_2 , which is then used in the peroxidative conversion of ethanol to acetaldehyde catalyzed by catalase.

In conclusion, although most studies have involved the use of non specific enzyme inhibitors to differentiate between the various enzyme reactions involved, the above observations suggest that MEOS activity is probably the result of NADPH oxidase and catalase activity.

The quantitative role in vivo of MEOS is unclear, as it cannot account for all of the increase in ethanol clearance rate shown in chronic ethanol treated rats³¹. Studies on the in vivo significance of MEOS have again depended on the use of non specific inhibitors. In most studies ADH activity was unchanged, and little work has been done on the quantitative significance of MEOS in experimental and pathological conditions where ADH activity is decreased.

Factors Affecting the Rate of Ethanol Metabolism

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(a) Ethanol Concentration. Widmark⁴⁶ has shown that ethanol is eliminated at a constant rate over a wide range of concentrations, the rate only decreasing when blood ethanol concentrations have fallen to about 2mM(9mg/100ml) in humans⁴⁶, and 4-2mM(18-9mg/100ml) in dogs⁴⁸. These concentrations are close to the Michaelis constant of isolated purified ADH, therefore ADH activity is thought to be responsible for this steady rate of ethanol elimination.

(b) Changes in the Rate of the ADH Reaction. A number of factors may influence this reaction, (i) the activity of ADH, (ii) the rate at which the mitochondrial shuttle system can transport reducing equivalents from NADH, produced by ADH, into the mitochondria, (iii) the rate of oxidation of NADH in the mitochondria.

(i) Activity of ADH. Decreases in ADH activity produced by inhibition with pyrazole,²² lead to decreased rates of ethanol clearance in rats²²; although subjects possessing the more active atypical form of ADH have normal rates of ethanol clearance⁴⁹. The activity of ADH is therefore not the sole determining factor which regulates the ADH reaction.

(ii) Transfer of Reducing Equivalents. More than 90% of the oxygen uptake of liver occurs in the mitochondria. NADH cannot penetrate the mitochondrial membrane and a carrier or shuttle system is used to transfer the reducing equivalents from NADH into the mitochondria. The malate - aspartate system has been postulated as the shuttle system responsible, as malate dehydrogenases are present in both the cytoplasm and mitochondria. Oxaloacetate is reduced to malate in the cytoplasm, and malate is then transported into the mitochondria and reoxidized to oxaloacetate. One

equivalent of NADH is thus produced inside the mitochondria. As⁵⁰
oxaloacetate permeates the mitochondrial membrane at a slow rate, this
system has been slightly altered to incorporate an intermediary⁵¹
transaminase system (Fig 2). Transaminase inhibitors have been used to
study the involvement of the malate - aspartate shuttle during ethanol
metabolism. The administration of transaminase inhibitors reduced the
ethanol elimination rate by 50%, as well as preventing the passage of⁵²
reducing equivalents into the mitochondria. Under normal conditions this
system is not rate limiting during ethanol metabolism, as the NADH
concentration inside the mitochondria increases by the same proportion as
that in the cytoplasm. NADH added to the complete system is oxidized at a
rate similar to that of ethanol oxidation in vivo. The mitochondrial
shuttle system can be induced after chronic ethanol treatment, as Rawat and⁵³
Kuriyama have shown an increase in mitochondrial permeability to NADH
in chronic ethanol treated mice.

⁵⁴
(iii) Oxidation of NADH. Videla and Israel have shown that the
rate of ethanol metabolism by the liver depends on the rate of mitochondrial
reoxidation of NADH, produced during ethanol oxidation. This was
demonstrated using uncouplers of mitochondrial oxidative phosphorylation,
such as 2,4 - dinitrophenol or arsenate, which increased the rate of
ethanol metabolism of normal rat liver slices, indicating that NADH
reoxidation was in fact rate limiting.

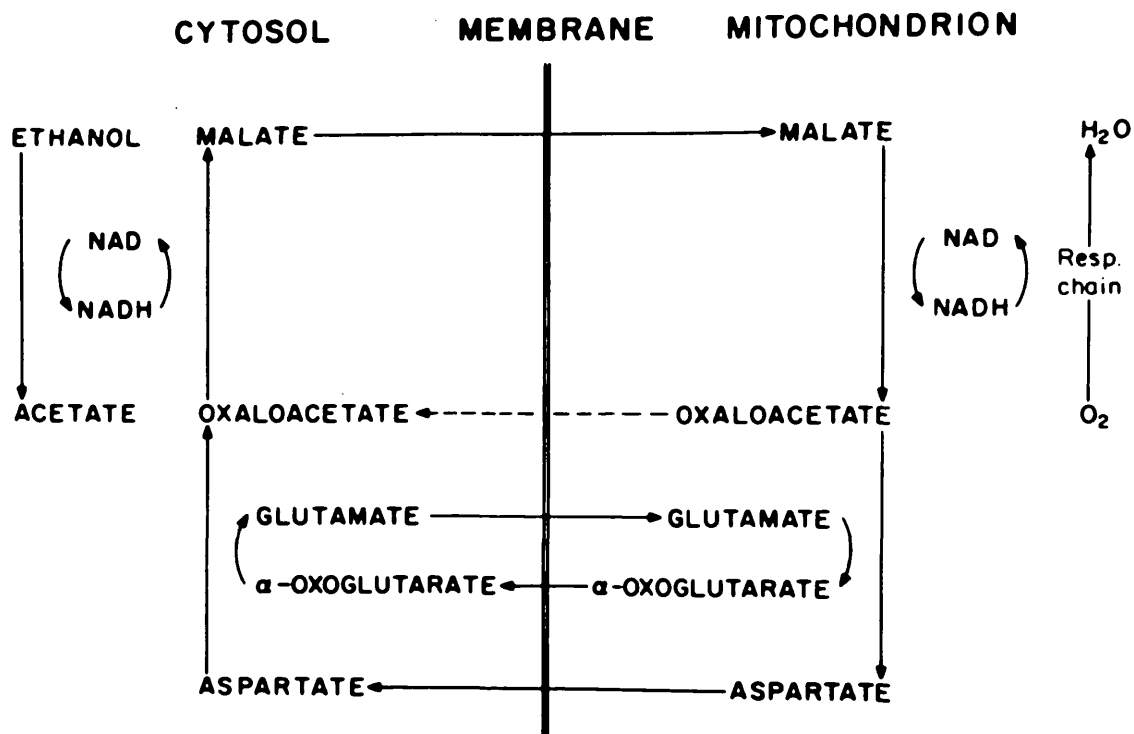


FIG. 2

Mitochondrial shuttle system for the transfer of reducing equivalents, produced from the dehydrogenation of ethanol in the cytosol, into the mitochondria.

Effect of Ethanol on Metabolic Processes

It is important to differentiate between metabolic effects secondary to the physiological action of ethanol and those produced by its metabolism. The metabolism of ethanol leads to the formation of a large amount of 2-carbon residues, which enter common metabolic pools and as a consequence can alter other metabolic pathways.

During ethanol metabolism the supply of cofactors, which are shared by other pathways involved in the oxidation of endogenous substances, is restricted. The hepatocyte undergoes a change from an oxidative to a reductive internal environment, which is reflected in the cytoplasmic, microsomal and mitochondrial compartments of the cell. This change in the redox state of the various cellular compartments influences many metabolic pathways.

Changes in NAD^+ Concentration. The increase in the ratio of the reduced to the oxidised form of NAD in the liver, which takes place after ethanol ingestion, can be demonstrated with very small concentrations of ethanol. Direct determination of NAD^+ and NADH in extracts from liver tissue showed a definite increase in NADH, although the total amount of the coenzyme did not change⁵⁵. The initial reduction of NAD^+ occurs within the cytoplasmic compartment, where ADH is located.

As NAD^+ and NADH exist in both free and bound forms, the amount of free coenzyme must be measured indirectly. This is done by measuring certain metabolites which form redox pairs, and are assumed to be in equilibrium with the free NAD^+ and free NADH of the cell. Lactate and pyruvate are used as a measure of the cytoplasmic free NAD^+ /free NADH ratio,⁵⁶ which is termed the redox state. Similarly the measurement of 3-hydroxybutyrate and acetoacetate can give a measure of the intramitochondrial redox state.

Tricarboxylic Acid Cycle. The presence of ethanol causes a marked

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depression of the rate of CO_2 formation from the liver. Forsander ,
using liver slices, showed that the respiratory quotient (RQ) decreased
from 0.74 to 0.02 when ethanol was present, without there being any
change in the oxygen uptake of the liver. It is possible to differentiate
between CO_2 production from the TCA cycle and that produced from other
cycles using radioactive labelled glucose. Using this technique,
58
Williamson et al have demonstrated that the TCA cycle is inhibited by
75% during ethanol metabolism. This is thought to be due to the
inhibition of isocitrate dehydrogenase by the presence of increased amounts
of NADH produced during ethanol metabolism.

Carbohydrate Metabolism. A major function of the liver is the

regulation of blood glucose concentration. The production of glucose from
pyruvate and other metabolites is known as gluconeogenesis. Krebs et al
59
have shown that ethanol inhibits gluconeogenesis from lactate but not
from pyruvate. When lactate is the gluconeogenic precursor, the ethanol
induced redox state shift prevents the conversion of lactate to pyruvate
and gluconeogenesis from lactate is inhibited.

The influence of ethanol on blood glucose concentration is dependent
on the nutritional state of the organism; well fed and starved organisms
react in different ways. Under normal conditions in man, and in well
nourished animals, ethanol causes a slight increase in blood glucose
concentration. This hyperglycemia is probably due to an increase in the
breakdown of liver glycogen brought about by an increased release of
60
adrenaline by ethanol . In the starved organism the blood glucose
concentration is derived exclusively through gluconeogenesis in the liver.

Animals that have been starved for a prolonged period (2 to 3 days) show a pronounced fall in blood glucose concentration after ethanol administration⁶¹. In man this condition known as alcohol induced hypoglycemia may be a serious or even fatal consequence of ethanol consumption.

Lipid Metabolism. The accumulation of fat in the liver during ethanol metabolism is associated with the development of alcoholic liver disease. The majority of liver biopsy specimens from alcoholics show the presence of fatty infiltration. Unfortunately, no obvious relationship has been found to link the development of steatosis with necrosis and cirrhosis of the liver, as only about 10% of alcoholics develop cirrhosis⁶². Ethanol can influence lipid metabolism in several ways. The 75% reduction in the TCA cycle shown by Williamson et al⁵⁸, can affect the oxidation of lipids as they furnish a large part of the energy produced by the liver. Fatty acid synthesis may be directly accelerated by the increased extramitochondrial concentration of NADH and NADPH. Nikkilä and Ojala⁶³ have shown that the reduction of dihydroxyacetone phosphate by NADH leads to increased amounts of glycerophosphate in the liver after ethanol administration, and this may facilitate the esterification of free fatty acids to triglycerides. It is also suggested that a large dose of ethanol may produce a non specific stress situation which leads to mobilisation of depot fat⁶⁴. All these mechanisms can therefore give rise to increased amount of triglycerides in the liver.

Ascorbic Acid

The isolation of L-ascorbic acid was carried out by Zilva⁶⁵, who established the general properties of the vitamin. He showed that the vitamin, isolated from lemon juice by precipitation with lead acetate, was a nitrogen free substance with powerful reducing properties. The physiological activity appeared to be associated with this reducing power,⁶⁶ although freshly oxidized solutions still retained their activity. This anomaly was resolved by Tillmans et al⁶⁷ who demonstrated that the vitamin could be reversibly oxidized to dehydroascorbic acid, and that both the oxidized and reduced forms were physiologically active. L-ascorbic or L-threo-hexono-1,4-lactone-2-ene is an optically active ($[\alpha]_D^{+23}$ in water), white crystalline solid melting at 192°C. It reacts as a monobasic acid⁶⁸ with a $pK_{a1} = 4.25$ in water, although a second ionisation constant⁶⁹ $pK_{a2} = 11.79$ has also been reported. It has been suggested that the in vivo role of ascorbic acid is related to its ease of oxidation and reduction, as both the reduced and oxidised forms are found in tissues with high metabolic activity. A role in electron transport has therefore⁷⁰ been suggested.

Collagen Formation. The formation and maintenance of normal collagen requires L-ascorbic acid; if ascorbic acid is absent a non-fibrous collagen precursor is formed instead of fibrous collagen.⁷¹ Robertson has suggested that ascorbic acid is required for the hydroxylation of proline to hydroxy-proline and this is then involved in the synthesis of collagen. It is thought that the hydroxylation of proline involves the formation⁷¹ of a free hydroxyl radical from ascorbic acid.

Action of Enzymes. Ascorbic acid can activate enzymes such as liver⁷² esterase⁷³ and catalase⁷⁴, but inhibits urease from plants. The

inhibition of urease by ascorbic acid takes place when copper ions are present; Cu^{3+} is reduced to Cu^{2+} by ascorbic acid and this ion has a greater affinity for the -SH groups of the enzyme⁷⁴. Ascorbic acid has no effect on enzymes when metal ions are absent, but it can enhance the inhibitory power of metal ions when they are present.

Drug Metabolism. The activity of liver microsomal drug metabolizing enzymes is influenced by many factors such as age, sex, strain and species, as well as the nutritional state of the animals. With respect to nutrition, the function of ascorbic acid in maintaining drug metabolism has been widely studied. Most early studies were in vivo investigations, which showed decreased metabolism of a large number of drugs in vitamin C deficient (scurbutic) animals. More recently the underlying biochemical basis of this decrease in drug metabolism in vitamin C deficiency has been studied.

⁷⁵
As early as 1941 Richards et al demonstrated an increase in pentobarbitone sleeping time in scorbutic guinea pigs, compared with normal controls. The administration of ascorbic acid was found to return the pentobarbitone sleeping time of the scorbutic guinea pigs to normal values. Axelrod et al⁷⁶ also showed a significant increase in the plasma half life of acetanilide, aniline, and antipyrine in scorbutic guinea pigs. It was therefore established that the rate of metabolism of various drugs was markedly reduced in vitamin C deficiency, but the biochemical basis for this decrease was unknown.

⁷⁷
In 1961 Conney et al observed that, guinea pigs fed on a vitamin C deficient diet for 10 - 14 days, and showing no obvious signs of scurvy, were sensitive to the muscle relaxant, zoxazolamine. The increased duration of this drug in vivo could be explained by a decrease in its oxidation, as liver microsomes prepared from scorbutic guinea pigs showed

decreased rates of oxidation of the drug in vitro. Leber et al , in 1969, demonstrated that liver microsomes from scorbutic guinea pigs showed significant decreases in the demethylation of aminopyrine, hydroxylation of acetanilide, and cytochrome P-450 content. There was no change in cytochrome b₅ content. The microsomal enzyme inducers phenobarbitone and 3- methylcholanthrene, were also shown to induce the activity of the mixed function oxygenases in scorbutic guinea pigs. These workers also showed that the prior administration of ethionine could block this induction of mixed function oxygenase activity. These results indicate that the system for de novo synthesis of microsomal enzyme protein is operable in the scorbutic guinea pig.

MATERIALS AND METHODS

Materials

Ascorbic Acid B.P. for administration to animals was supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks, England. Ethanol solutions for animals were made up with Absolute Alcohol B.P. 99.9% v/v, supplied by James Burrough Ltd., Fine Alcohols Division, 60 Montford Place, London S.E.11. n-Propanol was supplied by Reeve Angel Scientific Ltd., 14 New Bridge Street, London E.C.4, from their chromatographically tested range. Phenobarbitone B.P. as the sodium salt (Macarthys Ltd., Chesham House, Chesham Close, Romford RM1 4JX, Essex) and Phenytoin (Parke-Davis) were supplied by the pharmacy, Stobhill General Hospital, Glasgow. Enzymes and coenzymes were supplied by the Boehringer Corporation (London) Ltd., Bilton House, Uxbridge Road, Ealing, London, W5 2TZ. Porapak Q for gas-liquid chromatography was obtained from Waters Associates Inc. Framingham, Massachusetts, U.S.A. All other reagents were 'Analar' grade, and were supplied by B.D.H. Chemicals Ltd., Poole, England.

Methods

Vitamin C. Deficient Diet

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The diet used was based on that of Woodruff et al and had the following percentage composition, oat flakes 39; dried skimmed milk 30; wheat bran 20; vegetable oil 8; cod liver oil 2; and NaCl 1. Additions to this diet were made to improve its mineral and vitamin content. These additions were similar to those made by Ginter⁸⁰, and Hughes and Hurley⁸¹ who used diets with similar percentage compositions as that of Woodruff et al⁷⁹. The diet was supplemented with 0.5g MgO and 0.5g salt mixture/100g diet as recommended by Hughes and Hurley⁸¹ and Ginter⁸⁰ respectively.

The salt mixture of Ginter⁸⁰ contained (g), CaCO_3 60; K_2HPO_4 64.5; NaCl 33.5; MgSO_4 20.4; CaHPO_4 15; ferric citrate 5.5; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 1.0; KI 0.16; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.06; and ZnCl_2 0.05.

80

The following vitamin supplement, again as described by Ginter⁸⁰, was also added/100g diet, nicotinamide 20mg; calcium pantothenic 3mg; thiamine hydrochloride 2mg; riboflavin 2mg; folic acid 2mg; and pyridoxine hydrochloride 1mg.

Each animal was given a weekly supplement of 0.05ml cod liver oil by intubation, and 10g of hay as described by Hughes and Hurley⁸¹.

When supplemented with L-ascorbic acid, the diet supported normal growth of guinea pigs. Without ascorbic acid the guinea pigs became scorbutic.

Measurement of Ascorbic Acid in Guinea Pig Liver

Total liver ascorbic acid was measured by the 2,4-dinitrophenylhydrazine method of Roe and Kuether⁸² as modified by Bessey et al⁸³. The advantage of this modified method is that a large number of samples can be analyzed at the one time. As ascorbic acid is stable in trichloroacetic acid, whether or not the extract is separated from the protein precipitate, deproteinised samples can be left overnight in a fridge. A second advantage of this method is that ascorbic acid is oxidized to dehydroascorbic acid by cupric ions present in the 2,4-dinitrophenylhydrazine-thiourea-copper sulphate reagent, and a separate oxidation step is therefore not required.

Principles and Specificity. Ascorbic acid is oxidized, by cupric ions, to dehydroascorbic acid which couples rapidly with 2,4-dinitrophenylhydrazine in 10N H_2SO_4 , forming a bis-2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid (Fig 3). This derivative forms a highly stable brownish-red colour when treated with 65% H_2SO_4 , and this can be measured spectrophotometrically.

The specificity of the method is based on several principles (a) the colour is only produced by 2,4-dinitrophenylhydrazine derivatives of 6-carbon and 5-carbon sugarlike compounds.

(b) the rate of coupling with dehydroascorbic acid is much faster than with sugars or other sugarlike compounds.

(c) interfering chromogen formation is avoided by working with fairly dilute solutions of tissue extracts, and by carrying out the coupling reaction at a relatively low temperature ($37^{\circ}C$).

(d) the coupling reaction is carried out in the presence of the reducing agent thiourea, which contributes to the specificity of the method.

The colour formed after the coupling with 2,4-dinitrophenylhydrazine

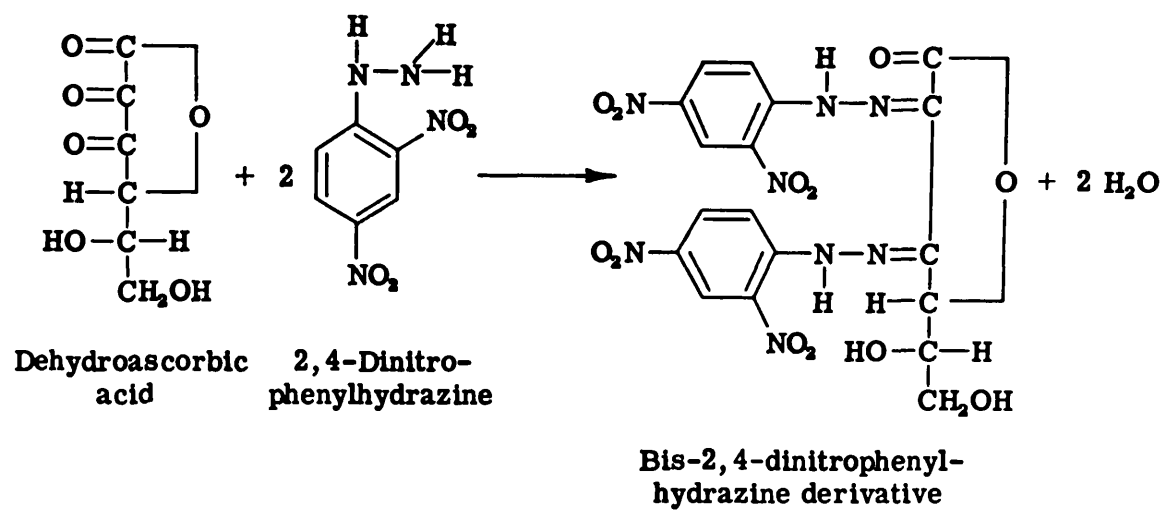


FIG. 3

Ascorbic acid coupling with 2,4-dinitrophenylhydrazine.

and treatment with 65% H_2SO_4 , is due to a molecular rearrangement of the bis-2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. The colour absorbs maximally at 500-550nm and 350-380nm. The chemistry of the coloured product is unknown, although it is established that it is produced by dehydrating conditions in a medium containing excess reducing agent. H_2SO_4 acts as both solvent and reactant, forming a very stable reddish-brown product. Most potentially interfering substances (e.g. glucose, fructose, and pentoses) form a coloured product in H_2SO_4 , but the colour fades in this reagent. After the addition of 65% H_2SO_4 the sample should therefore be left for 30 minutes before reading on a spectrophotometer.

Reagents

(1) 2,4-dinitrophenylhydrazine reagent containing 100 vols 2.2% 2,4-dinitrophenylhydrazine in 10N H_2SO_4 , 5 vols 5% thiourea and 5 vols 0.3% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

(2) 10% trichloroacetic acid (TCA).

(3) 5% trichloroacetic acid.

(4) 65% H_2SO_4 . To 30ml distilled water, add cautiously 70ml concentrated H_2SO_4 .

(5) Ascorbic acid standards. 100mg ascorbic acid is dissolved in 100ml 5% TCA, giving a concentration of 1000 $\mu\text{g}/\text{ml}$. This is diluted with 5% TCA to give 10, 5, 2.5, 1.0 and 0.5 $\mu\text{g}/\text{ml}$ solutions.

Method. 1g of liver is homogenised in 2ml distilled water (1 in 3 dilution).

1ml of the resulting homogenate is added to 19ml distilled water

(1 in 20 dilution). To 5ml of this diluted homogenate is added 5ml of 10% TCA (1 in 2 dilution). The final concentration of TCA is 5% and the final dilution of the tissue is 1 in 120. At this stage the tissue extract can be left overnight. The precipitated homogenate is centrifuged to remove cell debris and denatured protein, and 1ml of the resulting supernatant

is incubated with 0.3ml of the 2,4-dinitrophenylhydrazine-thiourea-copper sulphate reagent for 4 hours at 37°C. After cooling in an ice bath, 1.5ml of 65% H_2SO_4 is added and the sample is left for 30 minutes. The optical density of the sample is then read at 520nm against a blank of 5% TCA treated in the same way as the sample. A calibration graph of 0.5, 1.0, 2.5, 5.0 and 10 μ g/ml is prepared using the ascorbic acid standards, which are treated in the same way as the sample. The concentration of ascorbic acid in the sample can then be obtained directly from the standard graph (Fig 4).

Calculation

The ascorbic acid content of liver tissue is calculated:

$$\text{concentration}(\text{graph reading } \mu\text{g/ml}) \times \text{dilution}(120) = \mu\text{g ascorbic acid/g wet weight liver.}$$

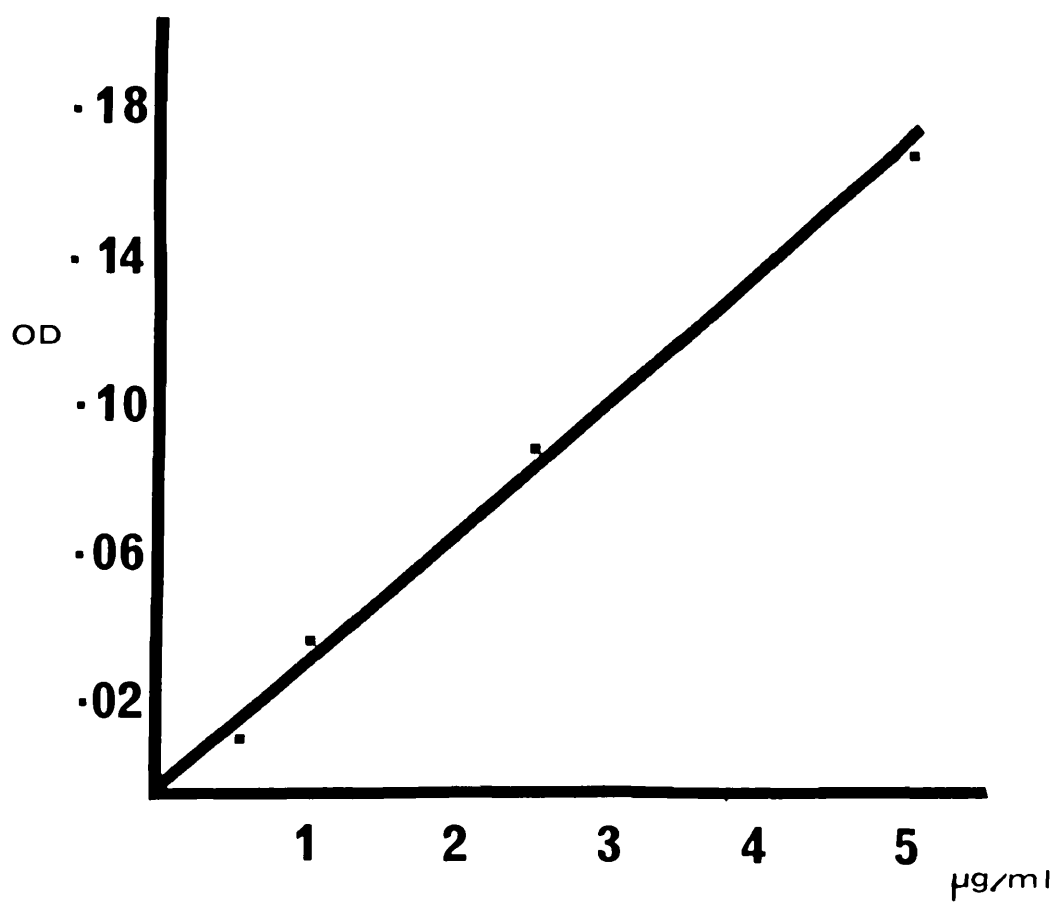


FIG.4 Ascorbic acid standard graph.

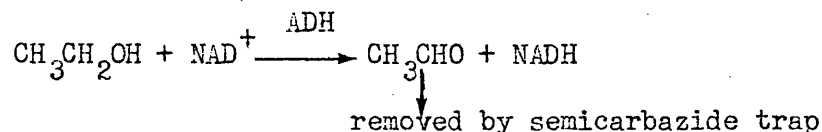
Alcohol Dehydrogenase Activity in Guinea Pig Liver

84

The method of Bonnichsen and Brink as modified by Hillbom and

85

Pikkarainen was used. This modification includes a semicarbazide trap for the acetaldehyde produced by the alcohol dehydrogenase (ADH) reaction. As acetaldehyde is being continually removed by this trap, the reaction can proceed at its maximum rate:



Reagents

- (1) 1% Triton X-100 in 0.25M sucrose.
- (2) Sodium pyrophosphate buffer (0.1M; pH9.0)

10g $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$

0.5g glycine

Dissolve in distilled water, adjust to pH9.0 with 1N HCl and make up to 300ml with distilled water.

- (3) Semicarbazide hydrochloride (2.2M)

Dissolve 5.0g semicarbazide HCl in 10ml 2N NaOH, adjust to pH6.3~6.5 with 5N NaOH and make up to 20ml with distilled water.

- (4) Ethanol (99.9%)

- (5) NAD (approx. 0.03M)

Dissolve 20mg NAD in 1ml distilled water.

Method. The guinea pig is killed by cervical dislocation and its liver removed and weighed. The following procedures are carried out at 4°C; 1g of liver is homogenised in 9ml of 1% Triton X-100 in 0.25M sucrose, using a teflon piston-glass homogeniser. R  ih   and Koskinen have previously demonstrated that this homogenising medium produces maximal ADH activities from liver. The homogenate is centrifuged for 30 minutes at

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10,000g, and the resulting supernatant is then centrifuged for a further 60 minutes at 100,000g in a Beckman L-2 ultracentrifuge. The supernatant obtained is used for the assay of ADH.

Assay

Experimental Cell

2.6ml pyrophosphate buffer
0.1ml semicarbazide solution
0.2ml NAD solution
0.02ml supernatant
0.1ml ethanol

Blank Cell

2.7ml pyrophosphate buffer
0.1ml semicarbazide solution
0.2ml NAD solution
0.02ml supernatant

Total volume = 3.02ml

Mix the cells and record the difference in their optical density at 340nm for 3 minutes, at 25°C, on a Pye Unicam SP8000 split beam recording spectrophotometer. The blank cell is used to eliminate the background production of NADH by the supernatant. The increase in optical density at 340nm, due to the formation of NADH, is therefore a measure of ADH activity of the supernatant.

Calculation

$$\frac{\Delta E / \text{min} \cdot V}{\epsilon \cdot d \cdot v} = U / \text{ml supernatant}$$

where

$\Delta E / \text{min}$ = optical density difference/min

V = assay volume = 3.02

ϵ = extinction coefficient of NADH (6.22cm² / μ mole at 340nm)

d = light path in cm = 1

v = volume of supernatant = 0.02ml

$$\therefore \Delta E / \text{min} \times 24.28 = U / \text{ml supernatant}$$

for the specific activity: $\frac{\Delta E / \text{min} \times 24.28}{\text{mg protein/ml supernatant}} = U / \text{mg protein}$

(1 enzyme unit is equal to 1 μ mole of NADH produced per minute at 25°C)

Freeze Clamping of Liver and Extraction of Metabolites

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The method of Williamson et al was used, the liver being prepared as follows. The guinea pigs are killed by cervical dislocation and their livers rapidly removed and pressed between aluminium tongs precooled in liquid nitrogen. The frozen liver is pulverised in a mortar to a fine powder, and liquid nitrogen is added frequently to keep the powder frozen. The powder is transferred to a weighed plastic centrifuge tube containing 2ml of frozen 30% (w/v) perchloric acid (HClO_4). After a rapid reweighing, the tissue (1-2g) is mixed with HClO_4 , care being taken that no thawing occurs. Ice cold distilled water (5ml) is added and the mixture is immediately homogenised in the centrifuge tube. This is continued for about 2 minutes until thawing is complete. Protein is removed by centrifuging the preparation for 10 minutes at 30,000g, and the supernatant is adjusted to pH 5-6 with 1.4ml of 20% (w/v) KOH. After standing for 30 minutes in the cold, the precipitate of KClO_4 is centrifuged off. The resulting supernatant is used for the determination of lactate, pyruvate, acetoacetate and 3-hydroxybutyrate concentrations as described below.

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Pyruvate and acetoacetate concentrations are determined in the same cell, by the method of Mellanby and Williamson, lactate and 3-hydroxybutyrate concentrations are determined separately by the methods of Hohorst,

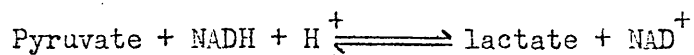
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and Williamson and Mellanby respectively.

Concentration of Pyruvate in Freeze Clamped Liver

Principle. Lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate with reduced nicotinamide adenine dinucleotide (NADH):



The equilibrium of the reaction is very much in favour of lactate formation. Provided there is an excess of NADH the reaction proceeds rapidly to completion, and pyruvate is quantitatively converted to lactate. The decrease in optical density at 340nm due to the oxidation of NADH is measured, and this is used in the calculation of the pyruvate concentration.

Assay

<u>Test cell</u>	<u>Control cell</u>
1ml phosphate buffer (0.1M;pH7.0)	1ml phosphate buffer
2ml supernatant	2ml water
0.1ml NADH approx 6mM (10mg NADH/2ml distilled water)	0.1ml NADH

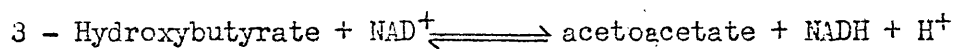
Wavelength 340nm, light path 1cm, final volume 3.1ml.

The test cell is initially read three times against the control cell, with a 3 minute interval between readings. LDH (0.001ml) is added to both cells, and their contents mixed. Readings at 3,6 and 9 minutes after the addition of LDH are then taken. The concentration of pyruvate in the freeze clamped liver is calculated as described below.

Concentration of Acetoacetate in Freeze Clamped Liver

Principle

3 - Hydroxybutyrate dehydrogenase catalyzes the reaction:



With a suitable excess of NADH, at least 98% of the acetoacetate is reduced to 3 - hydroxybutyrate, with the simultaneous oxidation of an equivalent amount of NADH. Again the decrease in optical density at 340nm due to the oxidation of NADH is measured.

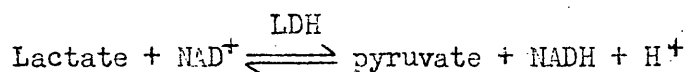
Assay

Acetoacetate concentration is measured at the end of the pyruvate assay, when a constant optical density is obtained. 3-Hydroxybutyrate dehydrogenase (0.025ml) is then added to both cells, and the optical density is read at 5 minute intervals until the reaction stops (approx. 20 mins). The concentration of acetoacetate in the freeze clamped liver is calculated as outlined below.

Concentration of Lactate in Freeze Clamped Liver

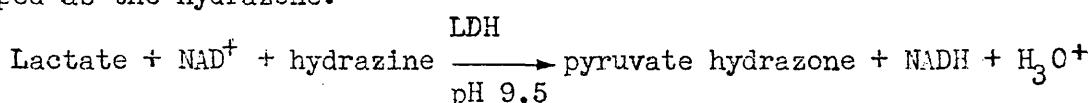
Principle

Lactate dehydrogenase (LDH) catalyzes the oxidation of lactate by NAD^+ :



As the equilibrium of the reaction lies far to the left, the reaction products must be removed to obtain quantitative oxidation of lactate.

Protons are bound by using an alkaline reaction medium, and pyruvate is trapped as the hydrazone:



The reaction is measured by recording the increase in optical density at 340nm due to the formation of NADH.

Assay

Test cell

1.4ml Hydrazine/glycine buffer

(0.4M hydrazine; 1M glycine; pH9.5)

0.1ml NAD (60mg/ml; approx. 50mM)

0.2ml supernatant

1.3ml H_2O

Control cell

1.4ml hydrazine/glycine buffer

0.1ml NAD

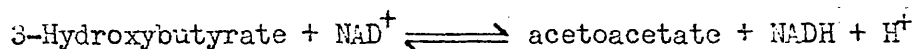
1.5ml H_2O

The cells are mixed and allowed to come to room temperature; the optical density is read three times with 3 minute intervals and 0.01ml LDH is added to the test cell. On completion of the reaction (10-20 minutes) the optical density is read a further three times with 3 minute intervals between readings. The concentration of lactate is calculated as described below.

Concentration of 3-Hydroxybutyrate in Freeze Clamped Liver

Principle

3-Hydroxybutyrate dehydrogenase catalyses the reaction:



In the presence of hydrazine, the acetoacetate is removed in the form of its hydrazone and the reaction proceeds quantitatively from left to right. The increase in optical density at 340nm due to the formation of NADH is a measure of the reaction.

Reagents

1. Tris buffer (0.1M; pH 8.5)

Dissolve 1.21g tris-hydroxymethyl-aminomethane in 50ml distilled water, add 14.3ml 0.2N HCl and make up to 100ml with distilled water.

2. Hydrazine buffer (pH 8.5)

Mix 1ml of hydrazine hydrate (99-100%) and 5ml 1N HCl and dilute to 20ml with distilled water.

3. Nicotinamide adenine dinucleotide (approx 13mM)

Dissolve 20mgNAD in 2ml distilled water.

Assay

Test cell

1.0ml hydrazine hydrate buffer

0.5ml tris buffer

1.5ml supernatant

0.1ml NAD

Control cell

1.0ml hydrazine hydrate buffer

0.5ml tris buffer

1.5ml distilled water

0.1ml NAD

Mix the cells and read the optical density against the control cell three times with three minute intervals between readings. Add 0.025ml

3-hydroxybutyrate dehydrogenase(Boehringer Corp) and read the optical density at 10 minute intervals until the reaction stops (40-60 minutes).

The calculation of the concentration of 3-hydroxybutyrate in freeze clamped liver is described below.

Calculation of Concentrations of Metabolites

In the determination of these metabolites, the optical density occasionally changes slowly at the end of the reaction. The reason for this 'background creep' is that the enzymes used for the determination of the metabolite concentrations may contain contaminating enzymes, which slowly react with other substances contained in the supernatant. This creep in optical density, which is linear with time, is superimposed on the entire reaction. It can be corrected for by graphically extrapolating to t_0 (the time of the start of the reaction). Readings of the optical density are taken at three minute intervals, these are plotted against time, and the linear part of the curve is extended backwards to time t_0 . The point where it cuts the ordinate axis gives the correct end point of the reaction, and therefore the correct optical density change ΔE (Fig 5). Extrapolation to t_0 was carried out for each of the metabolites measured. No correction was made for the % yield of metabolites from the freeze clamped liver extracts.

Calculation.

$$\frac{\Delta E \times V}{\epsilon \times d \times A} = \mu\text{moles metabolite/g wet weight liver.}$$

where

ΔE = corrected optical density change

V = volume of the assay mixture (ml)

A = portion of tissue (g)

= g tissue taken $\frac{\text{X ml supernatant used for assay}}{\text{total ml supernatant}}$

ϵ = extinction coefficient of NADH (6.22 cm $\frac{1}{\mu\text{mole}}$ at 340nm)

d = light path of cell = 1cm

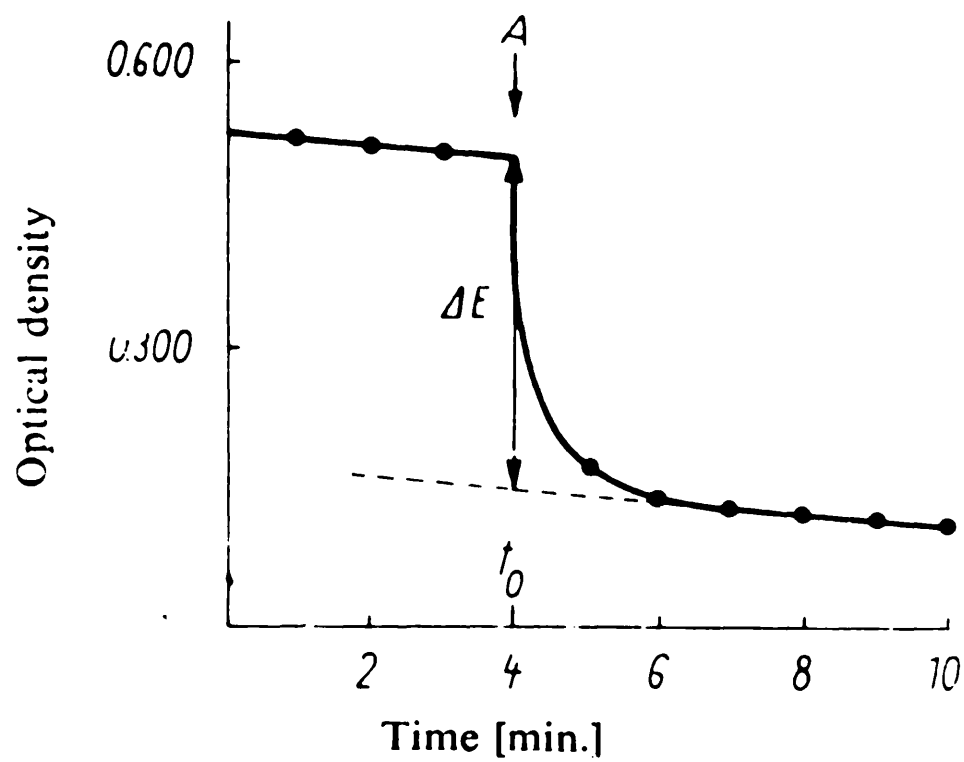


FIG. 5

Extrapolation to t_0 for determination of metabolites.

A: start of reaction with enzyme.

Calculation of NADH / NAD⁺ Ratios

The ratio of the concentrations of free NADH and NAD⁺ $\left(\frac{[NADH]}{[NAD^+]}\right)$ can be measured using the ratio of the concentrations of the oxidized and reduced metabolites of suitable NAD- linked dehydrogenase systems which, because of their high activity, are in equilibrium with the free nucleotides, according to the equation:

$$\frac{\left[\text{Oxidized substrate} \right] \left[\text{NADH} \right]}{\left[\text{Reduced substrate} \right] \left[\text{NAD}^+ \right]} = K$$

If K is known, the $\frac{[NADH]}{[NAD^+]}$ ratio can be calculated from the concentrations of the substrates. The lactate- pyruvate system can give reliable values for the cytoplasmic $\frac{[NADH]}{[NAD^+]}$ ratio, and the 3-hydroxybutyrate-acetoacetate system gives a measure of the intramitochondrial $\frac{[NADH]}{[NAD^+]}$ ratio ⁸⁷.

$$\text{Cytoplasmic } \frac{\{NADH\}}{\{NAD^+\}} = \frac{\{Lactate\}}{\{pyruvate\}} \times 1.11 \times 10^{-4}$$

$$\text{and Intramitochondrial } \frac{\{NADH\}}{\{NAD^+\}} = \frac{[3-Hydroxybutyrate]}{[acetoacetate]} \times 4.93 \times 10^{-2}$$

The values of the equilibrium constants for the lactate-pyruvate and 3-⁸⁷ hydroxybutyrate-acetoacetate systems were obtained from Williamson et al .

Preparation of Guinea Pig Liver Microsomes

The animals are killed by cervical dislocation and their livers perfused in situ with ice cold isotonic saline, to remove as much blood as possible from the tissue. All further procedures are carried out at 4°C. The livers are quickly removed, blotted and weighed, and 1 in 4 homogenates in 1.15% KCl are prepared using a teflon piston glass homogeniser. The homogenate is centrifuged for 10 minutes at 900g to remove cell walls and nuclei, and the resulting supernatant is centrifuged at 20,000g for 15 minutes to remove mitochondria. The microsome containing supernatant, which is produced, is centrifuged at 105,000g for 60 minutes; the resulting microsomal pellet is washed, resuspended in 1.15% KCl, and recentrifuged for a further 60 minutes at 105,000g. The final washed microsomal pellet is suspended in 1.15% KCl to give a concentration of 6mg protein/ml, and this suspension is used in the following microsomal enzyme assays, and estimations of microsomal electron transport components.

Estimation of Microsomal Electron Transport Components

Cytochrome P-450 and cytochrome b_5 were measured in the same cell
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by the method of Omura and Sato .

Assay

2ml of phosphate buffer pH7.0 (0.15M) and 1ml of microsome suspension (6mg protein/ml) are added, in duplicate, to a test and reference cell, giving a final concentration of 2mg microsome protein/ml in the cells. The base line difference spectrum of the two cells is recorded between 400 and 550nm in a Unicam SP8000 dual beam recording spectrophotometer. All spectrophotometric measurements are carried out at room temperature.

Cytochrome b_5 .

5 μ l of a 0.2mM NADH solution is added to the test cell to reduce cytochrome b_5 . The difference spectrum between the test and reference cell is then recorded in the range 400 to 500nm. The same cells are used in the following assay.

Cytochrome P-450

CO is bubbled through the test cell for 20 seconds, 5mg of sodium dithionite is then added to both cells and CO is bubbled through the test cell for a further 20 seconds. The difference spectrum is recorded in the range 400 to 500nm.

Calculation of Electron Transport Component Concentrations

Cytochrome P-450 Concentration

$$\frac{\Delta E \times V}{\epsilon \times d \times v \times c} \times 1000 = \text{nmoles cytochrome P-450/mg microsomal protein}$$

where

ΔE = difference in optical density between 450 and 490nm of difference spectrum

ϵ = molar extinction coefficient between 450 and 490nm = $91\text{cm}^{-1} \text{mM}^{-1}$

d = light path of cell = 1cm.

V = total volume of cell = 3ml.

v = volume of microsome suspension taken = 1ml

c = protein concentration of microsome suspension = 6mg/ml

Cytochrome b_5 Concentration

The difference in optical density between 423 and 500nm of the difference spectrum, divided by the concentration of protein/ml in the cell (2mg/ml) is used to express cytochrome b_5 concentration.

Thus:-

$$\frac{\Delta E_{423-500\text{nm}}}{2} = \Delta E_{423-500\text{nm}} / \text{mg microsomal protein}$$

Aniline Hydroxylase Assay

Microsomal aniline hydroxylase activity was determined by the method of Holtzman and Gillette⁹². The assay is performed in duplicate in 20ml serum bottles containing:

0.5ml Tris-HCl (150 μ moles, pH7.4 at 37°C)

0.5ml of a solution containing:

MgCl₂ (15 μ moles)

glucose-6-phosphate (30 μ moles)

NADP (1.5 μ moles)

glucose-6-phosphate dehydrogenase (2 units)

1.0ml microsome suspension (6.mg protein/ml)

The serum bottles containing the above solutions are preincubated for 5 minutes at 37°C, 1ml of 3mM aniline hydrochloride is then added, and the reaction incubated for 10 minutes at 37°C. Under these conditions the reaction is linear with respect to time and protein concentration. The reaction is stopped by placing the bottles in an ice bath. The amount of p-aminophenol formed during the reaction is determined by adding a 2.6ml portion of the incubation medium to a 45ml stoppered tube containing 25ml diethyl ether and 1g of solid NaCl. The tubes are then shaken vigorously by hand. A 20ml portion of the ether phase is transferred to a second 45ml stoppered tube and 1.6ml of 1% aqueous phenol, followed by 0.4ml of 0.5M K₃PO₄ is then added. The tubes are shaken, the colour allowed to develop for 20-30 minutes, and the optical density of the aqueous phase is read at 650nm against a blank. The blank is prepared from an assay which contains 1.0ml of 1.15% KCl instead of the microsome suspension. It is extracted in the same manner as described above, and the aqueous phase is added to a cell which is used as the blank. The aqueous p-aminophenol samples are read against this blank cell.

A standard graph of optical density against p-aminophenol concentration is obtained in the following manner. Solutions of p-aminophenol are prepared so that 1.0ml contains 25, 50, 75 and 100 nmoles of p-aminophenol. 1.0ml of each of the prepared p-aminophenol solutions is diluted with 2.0ml of water and is extracted in the same manner as described above for the incubation medium. A standard graph is constructed by plotting the optical density of the p-aminophenol standards against the number of nmoles of p-aminophenol they contain/ml. As the standards are diluted and extracted in the same manner as for the assay samples, the amount of p-aminophenol produced in each assay can be obtained directly from the standard graph (Fig 6). The standard graph does not go through the origin because of the slight absorbance of the blank used. Aniline hydroxylase activity is expressed as nmoles p-aminophenol produced/mg microsomal protein/hour at 37°C.

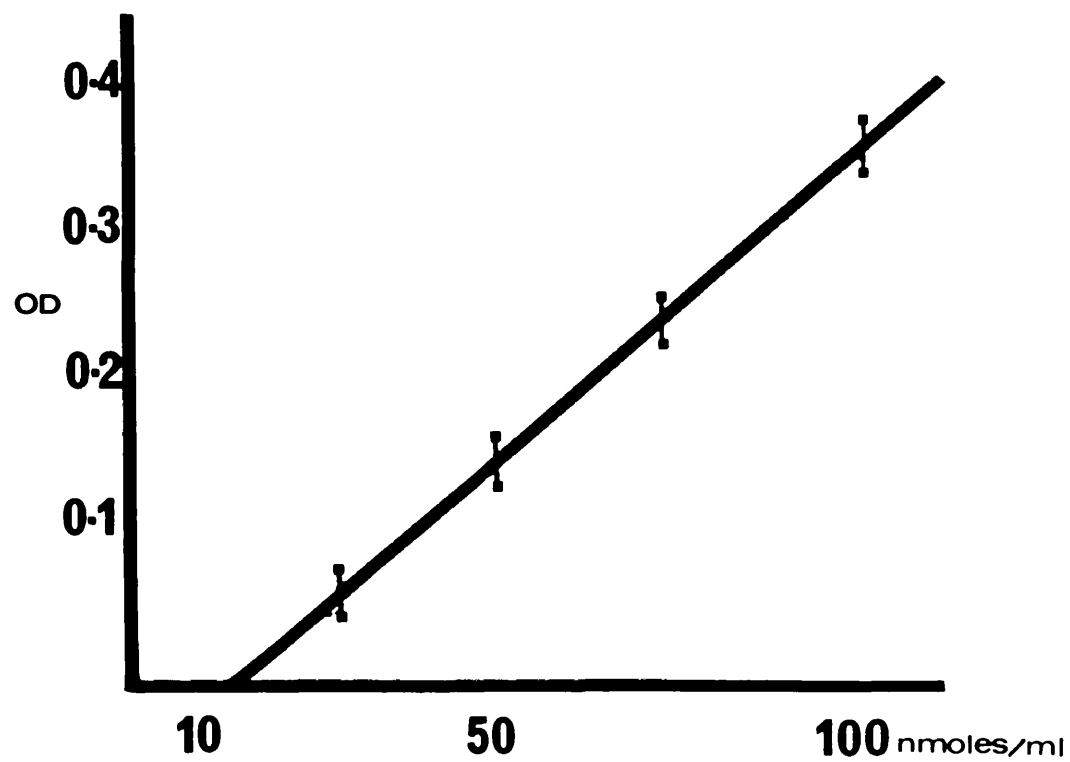


FIG. 6 Standard graph of p-aminophenol

MEOS activity was determined by the method of Leiber and De Carli .

In this assay the activity of MEOS is determined by measuring the amount of acetaldehyde produced. Acetaldehyde is trapped by a semicarbazide system, and the optical density of the resulting acetaldehyde-semicarbazone complex gives a measure of the amount of acetaldehyde produced by MEOS.

Assay

0.2ml phosphate buffer pH7.4 (80 μ moles)

0.5ml microsome suspension (equal to 3mg microsomal protein)

0.2ml NADPH generating system containing:

0.3 μ moles NADP

5.0 μ moles $MgCl_2$

20.0 μ moles Nicotinamide

8.0 μ moles Sodium isocitrate

0.01ml Isocitrate dehydrogenase (0.2 units)

The assay is carried out in duplicate in Thunberg tubes which contain 0.6ml of 0.015M semicarbazide HCl in 0.16M phosphate buffer pH7.0 in their side arms. Thunberg tubes containing the above assay solutions are preincubated for 5 minutes at 37°C, 0.1ml ethanol solution (50 μ mole) is then added to each tube and they are incubated for 10 minutes at 37°C.

The reaction is stopped by the addition of 0.5ml of 70% trichloroacetic acid. After an overnight diffusion at room temperature, the amount of acetaldehyde bound to the semicarbazide is determined by the method of

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Gupta and Robinson . A 0.2ml aliquot of the acetaldehyde-semicarbazone complex in the side arm is diluted to 1.0ml with water, and its optical density is read at 224nm against a blank cell. A reaction blank is prepared from the assay system described above except that 0.5ml of water is

added instead of the microsome suspension. An aliquot (0.2ml) of the solution in the side arm of this reaction blank is diluted to 1.0ml with water, and this is used for the blank cell.

Calculation.

Under the condition of the assay, 0.1 μ mole of acetaldehyde produced gives an optical density of 0.33.

$$\begin{aligned} \bullet & \quad \frac{\text{Optical density reading}}{0.33} \text{ is equivalent to } A \times 0.1 \mu\text{moles}/10\text{min}/3\text{mg protein} \\ \bullet \bullet & \quad \text{where } A = \frac{\text{optical density reading}}{0.33} \end{aligned}$$

$$\begin{aligned} A \times 0.1 \mu\text{moles}/10\text{min}/3\text{mg protein} &= \frac{0.A \mu\text{moles}}{30} / \text{min}/\text{mg protein} \\ &= \frac{1000 \times 0.A \text{ nmoles}}{30} / \text{min}/\text{mg protein} \\ &= \frac{A \times 10}{3} \text{ nmoles}/\text{min}/\text{mg protein} \\ \bullet & \quad \frac{\text{optical density reading} \times 10}{0.99} = \text{nmoles acetaldehyde produced}/\text{min}/ \\ \bullet \bullet & \quad \text{mg microsomal protein} \end{aligned}$$

NADPH Oxidase Assay

NADPH oxidase activity was measured spectrophotometrically in microsomal preparations by determining the rate of disappearance of NADPH at 340nm, as described by Gillette et al. ⁹⁴.

Assay

Experimental Cell

Blank Cell

0.5ml phosphate buffer (0.2M; pH7.4)

0.5ml phosphate buffer

0.3ml microsome suspension (6mg protein/ml)

0.3ml microsome suspension

1.0ml nicotinamide (100 μ moles)

1.0ml nicotinamide

0.2ml H₂O

1.2ml H₂O

The reaction is started by adding 1.0ml NADPH (0.25 μ moles) to the experimental cell, which is read at 340nm against the blank cell for 5 minutes at 25°C using a Unicam SP 8000 split beam recording spectrophotometer. The change in optical density/minute is used to calculate the activity of NADPH oxidase.

Calculation

$$\frac{\Delta E/\text{min} \times V \times 10^3}{\epsilon \times v \times d} \times \frac{1}{\text{mg protein/ml}} = \text{nmoles NADPH oxidised/minute/ mg microsomal protein}$$

where

$\Delta E/\text{min}$ = change in optical density/minute

V = total volume of cell = 3.0ml

ϵ = molar extinction coefficient = 6.22cm²/umole NADPH at 340nm

d = light path = 1cm

v = volume of microsome suspension taken = 0.3ml

Catalase Assay

Catalase activity of human liver supernatant, and guinea pig microsomal preparations was measured by the oxygen electrode method of Goldstein⁹⁵. The advantage of this method is that linear reaction rates can be obtained, as the reaction is only measured for 30 seconds and a negligible proportion of the substrate is used up. Perborate which furnishes H_2O_2 in solution is used as the substrate for catalase.

Method

Oxygen saturated distilled water (3.0ml) is added to the reaction chamber of the oxygen electrode which is maintained at 30°C . The chart recorder attached to the electrode is adjusted to give a 100% full scale reading for oxygen saturated distilled water. The distilled water is removed and 3.0ml of 0.033M sodium perborate in 0.05M sodium phosphate buffer pH7.0 is added to the reaction chamber. The perborate is equilibrated to 30°C , 10 μl of enzyme preparation is added to the reaction chamber, and the reaction is measured for 30 seconds. The oxygen concentration in the reaction chamber rises rapidly and at a constant rate for at least 30 seconds, before there is any decrease in the rate of production of oxygen. The specific activity of catalase is expressed as $\mu\text{moles O}_2$ evolved/minute/mg microsomal protein.

Calculation

$$\begin{aligned}\text{Solubility of O}_2 \text{ in water} &= 0.235 \mu\text{moles/ml at } 30^\circ\text{C} \\ &= 0.705 \mu\text{moles/3ml}\end{aligned}$$

$$100\% \text{ full scale reading} = 100 \text{ units} \equiv 0.705 \mu\text{moles O}_2$$

$$1 \text{ unit} \equiv 0.00705 \mu\text{moles O}_2$$

$$\text{If reaction rate} = A \text{ units/30 seconds}$$

$$= 2A \text{ units/minute}$$

$$\text{then } 2A \text{ units/minute} = 2A \times 0.00705 \mu\text{moles O}_2/\text{minute}$$

$$\therefore \mu\text{moles O}_2/\text{min/mg protein} = \frac{2A \times 0.00705}{\text{mg protein taken}}$$

Determination of Blood Ethanol by Gas-Liquid Chromatography

The internal standard gas-liquid chromatography (GLC) method of
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Cooper was used. This method involves precipitating blood proteins before injection of the sample onto the GLC column. As propanol is used as an internal standard, and the detector response is the same for propanol and ethanol, accurate quantitation can be made (Fig 7). The method of Cooper was modified so that 20 μ l blood samples, obtained from the ear vein of guinea pigs, could be used.

Apparatus and Operating Conditions

A Pye 104 GLC instrument is used with a 5 foot glass column packed with Porapak Q (80-100 mesh). The instrument is equipped with a flame ionisation detector. Column temperature 170°C; injection port temperature 200°C; carrier gas (nitrogen) flow rate 60ml/min; hydrogen flow rate 66ml/min and air flow rate 500ml/min.

Method

The ear vein is pricked with a fine needle and 20 μ l of blood is removed with a pipette which has been previously rinsed in heparin. The blood sample is added to 0.5ml of aqueous propanol (10mg/100ml) and the pipette is rinsed with the propanol to remove all traces of blood. 0.05ml sodium tungstate (10g/100ml) followed by 0.05ml of $\frac{2}{3}$ N sulphuric acid are added to the propanol to precipitate the blood proteins. The sample is centrifuged at 900g for 10 minutes to remove the precipitate, and 5 μ l of the supernatant is injected onto the GLC column for blood ethanol determination. A typical GLC trace obtained is illustrated in Fig 8. Peak areas are measured by multiplying the peak height by its width at half height. As the detector response is the same for ethanol and propanol, the following calculation holds:

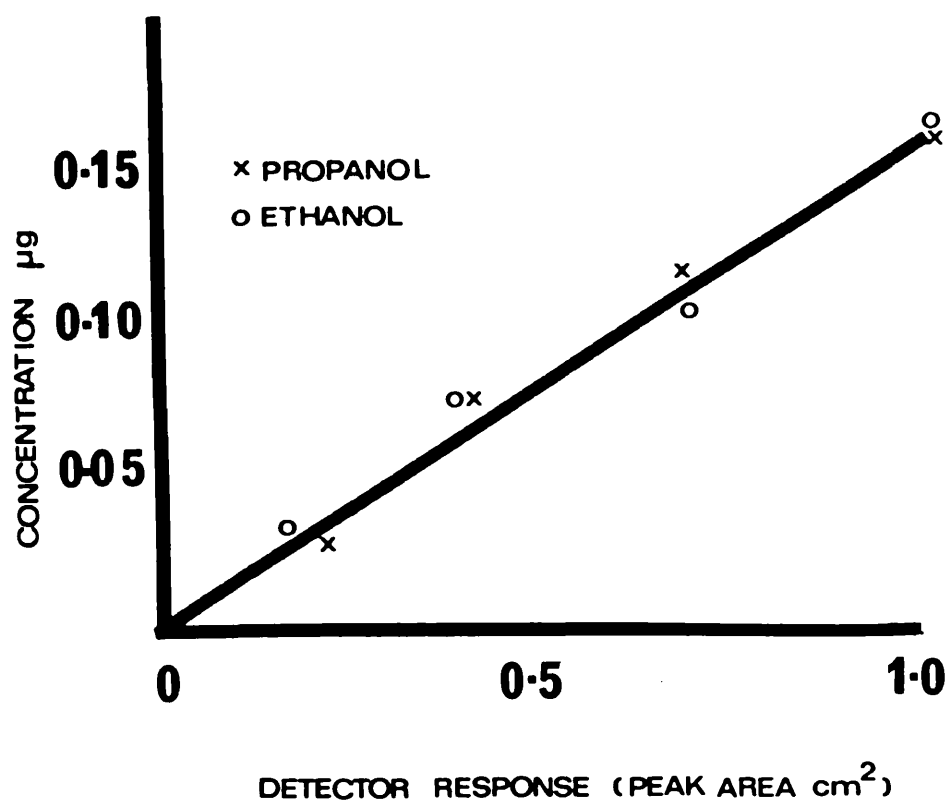


FIG. 7 GLC detector response to propanol and ethanol

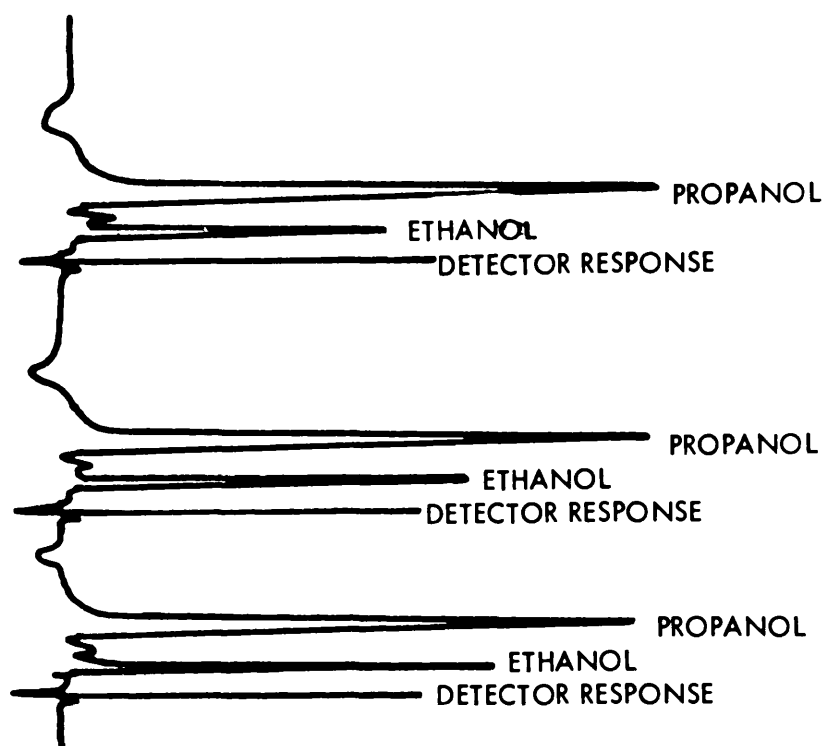


FIG. 8

A typical GLC trace of blood ethanol estimation.

Propanol standard = $10\text{mg}/100\text{ml} = 0.05\text{mg}/0.5\text{ml}$

∴ GLC sample contains 0.05mg propanol

When area of ethanol peak is equal to area of propanol peak

GLC sample contains 0.05mg ethanol

this 0.05mg originally in 0.02ml blood

5mg originally in 2ml blood

250mg originally in 100ml blood

∴ blood ethanol concentration = $250\text{mg}/100\text{ml}$ blood

For unknown sample

$$\frac{\text{Area ethanol peak}}{\text{Area propanol peak}} \times 250 = \text{blood ethanol concentration in mg ethanol}/100\text{ml blood}$$

Ethanol Clearance from the Blood

The rate of ethanol elimination from the blood, the amount of ethanol metabolized/Kg body weight/hour, C_0 (theoretical ethanol concentration at zero time, assuming complete absorption and uniform distribution), and r (fraction of body mass in which ethanol is equilibrated with the blood) were determined by the method of Widmark as described by Khanna and Kalant . 46

The rate of clearance of blood ethanol is obtained by plotting a graph of blood ethanol concentration against time (Fig 9). A straight line is constructed from the linear portion of this graph using the method of least squares. The slope of this straight line is equal to the rate of ethanol clearance from the blood, which is expressed in mg ethanol/100ml blood/hour.

Values for C_0 are obtained by extrapolating the straight line graph to zero time, C_0 is expressed in mg ethanol/100ml blood.

r is equal to:

$$\frac{A}{C_0 P} = \frac{\text{mg}}{\text{mg/litre} \times \text{Kg}} = \text{litre/Kg}$$

where A = total dose of ethanol in mg

C_0 = ethanol concentration at zero time mg/litre blood

P = weight of animal in Kg

The amount of ethanol metabolized mg ethanol/Kg body weight/hour is calculated:

$$\begin{aligned} & \text{mg ethanol eliminated/litre blood/hour} \times r \\ &= \frac{\text{mg}}{\text{litre} \times \text{hour}} \times \frac{\text{litre}}{\text{Kg}} = \text{mg ethanol/Kg body weight/hour} \end{aligned}$$

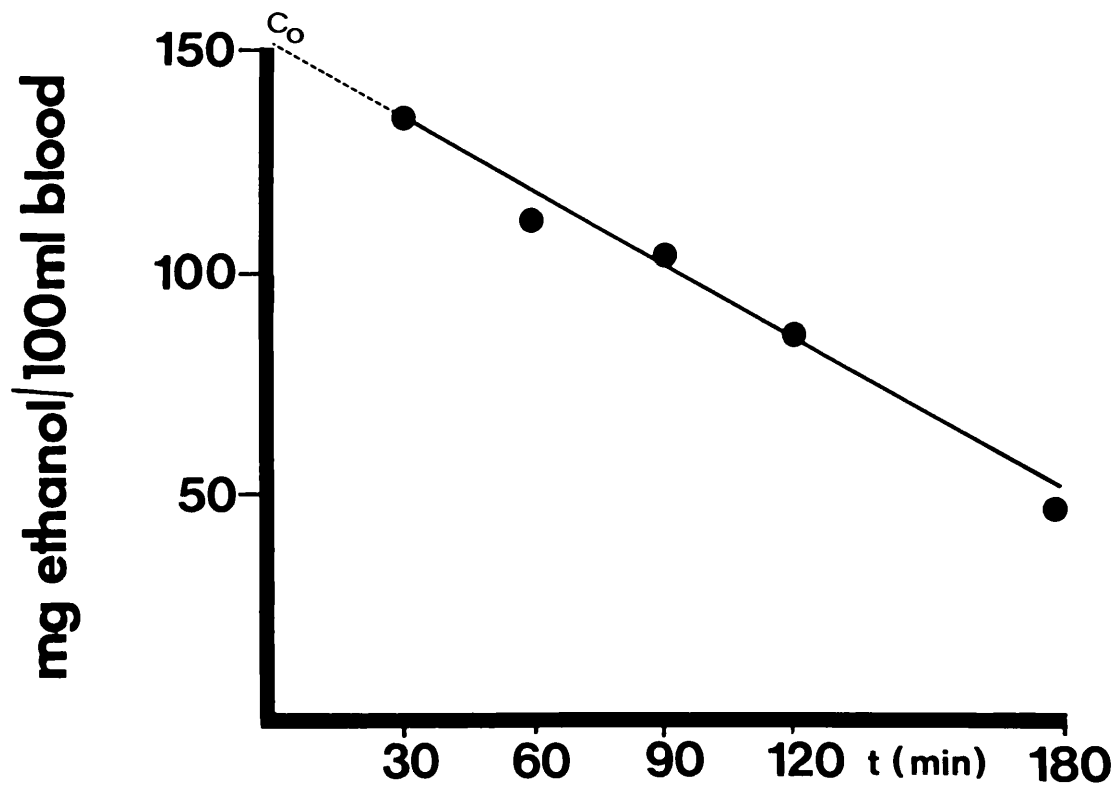


FIG. 9

Blood ethanol clearance against time. C_0 is theoretical ethanol concentration at zero time, assuming complete absorption and uniform distribution.

Serum $t_{\frac{1}{2}}$ of Diphenylhydantoin in the Guinea Pig

Animal treatment

Guinea pigs were fasted overnight and injected i.p. with diphenylhydantoin (25 mg/Kg). Blood samples were removed from the ear vein at 1, 2, 4, 6 and 24 hours after the injection. Serum was prepared in the normal manner and was used in the following radioimmunoassay for diphenylhydantoin estimation.

Radioimmunoassay of Diphenylhydantoin

The conventional radioimmunoassay procedure of Paxton⁹⁷ was followed. Standard displacement curves were produced by addition of increasing amounts of diphenylhydantoin to tubes containing trace amounts of ^3H - diphenylhydantoin and antiserum in phosphate buffer. Separation of bound and free label was achieved by addition of a donkey anti - rabbit precipitating serum. After incubation for 1 hr at 37°C the tubes were centrifuged and the supernatant containing the free label was removed for counting in a liquid scintillation spectrometer. The guinea pig serum was diluted 1 in 100 and was added to tubes containing trace amounts of ^3H - diphenylhydantoin and antiserum in phosphate buffer. The tubes were then treated as above, and the concentration of diphenylhydantoin in the serum was obtained from the standard displacement curve. All determinations were carried out in duplicate. The concentration of diphenylhydantoin in guinea pig serum was expressed in $\mu\text{g/ml}$.

$t_{\frac{1}{2}}$ of Diphenylhydantoin

The concentration of diphenylhydantoin in guinea pig serum was plotted on semilog graph paper against time. The $t_{\frac{1}{2}}$ was calculated from the linear portion of the graph.

Human ADH Assay

Preparation of Supernatant

All procedures are carried out at 4°C. The liver biopsy sample is homogenised in 0.25M sucrose containing 1% Triton X-100, this has been shown previously by Riih  and Koskinen⁸⁶ to give maximal ADH activities. A dilution of approximately 1 part tissue to 50 parts homogenising medium is used for both needle biopsy tissue and wedge biopsy tissue from surgery. The homogenate is centrifuged at 10,000g for 30 minutes, and the resulting supernatant fluid is used for the determination of ADH activity, as well as catalase and NADPH dependent ethanol oxidizing system activities.

ADH Determination

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The method of Von Wartburg et al¹⁸ was used as this can detect the presence of atypical ADH in biopsy samples by comparing the activity at pH8.8 with that at pH11.0. A modification of this method was carried out so that the final concentrations of NAD and ethanol were 0.5mM and 100mM respectively. These concentrations gave maximum and linear reaction rates for the time interval of the reaction with ethanol (3 minutes) and the concentration of enzyme preparation used.

Assay

pH 8.8

1.4ml sodium pyrophosphate buffer
(0.033M; pH 8.8)

0.05ml NAD
(final concentration 0.5mM)

0.05ml supernatant

pH 11.0

1.4ml glycine-NaOH buffer
(0.033M; pH 11.0)

0.05ml NAD
(final concentration 0.5mM)

0.05ml supernatant

The increase in optical density of each cell is recorded at 340nm on a Unicam SP 8000 spectrophotometer for 5 minutes, at 25°C against air. 0.05ml of ethanol (final concentration 100mM) is then added to both cells and

the increase in optical density is recorded for a further 3 minutes. As each cell acts as its own blank, the optical density change/minute without ethanol is subtracted from the optical density change/minute with ethanol. The specific activity of ADH is calculated as described for guinea pig ADH. The activity of ADH at pH 8.8 and pH 11.0 is expressed as units/g protein or units/g wet weight liver. One enzyme unit is equal to 1 μ mole of NADH produced per minute at 25°C.

NADPH Dependent Ethanol Oxidizing System

Activity in Human Liver

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The method of Mezey and Tobon was used, with the following modifications (a) 0.1ml of supernatant was added to the incubation mixture in a Thunberg tube to give a total assay volume of 1.0ml (b) 0.6ml of semicarbazide was added to the side arm of the Thunberg tube.

Principle

Acetaldehyde produced from ethanol by the enzyme reaction is trapped by semicarbazide to form an acetaldehyde-semicarbazone derivative. The concentration of this derivative is determined spectrophotometrically, and the amount of acetaldehyde produced by the reaction can be calculated.

Assay

0.6ml phosphate buffer (0.1M; pH 7.4)

0.1ml supernatant

0.2ml NADPH generating system containing (final concentrations)

NADP 0.3mM

MgCl₂ 5.0mM

Nicotinamide 5.0mM

Isocitrate dehydrogenase 1μM unit equivalent to 0.05ml

The assay is carried out in duplicate, in Thunberg tubes containing 0.6ml of 0.015M semicarbazide HCl in 0.16M phosphate buffer pH 7.0 in their side arms. The tubes containing the above assay solutions are preincubated at 37°C for 5 minutes. 0.1ml ethanol (115mM final concentration) is then added to the tubes, and the reaction is incubated for 10 minutes. The reaction is stopped with 0.5ml of 70% TCA, and the amount of acetaldehyde produced is determined using the same method as described for the MEOS system. A reaction blank containing all the assay solutions,

except that the supernatant is replaced with water, is also incubated. The semicarbazide solution in the side arm of this reaction blank is used to prepare a blank cell. The activity of the NADPH dependent ethanol oxidizing system is expressed as nmoles acetaldehyde produced/minute/mg of protein and nmoles acetaldehyde produced/minute/g wet weight liver.

Determination of Leucocyte Ascorbic Acid

Leucocyte ascorbic acid (LAA) was measured by the method of
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Denson and Bowers .

Reagents

(1) Diluent for blood

200ml physiological saline

50ml 6% dextran

2.0ml 10% sequestrene

This is prepared in bulk and distributed in 12.5ml amounts

(2) Reagent

100 vols 2.2% 2,4-dinitrophenylhydrazine in 10N H_2SO_4

5 vols 5% thiourea

5 vols 0.6% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

(3) 5% TCA

(4) 65% H_2SO_4

Approximately 3ml of blood is added to 12.5ml of diluent. The diluted blood is allowed to stand for half an hour at room temperature to allow the red cells to sediment. The supernatant fluid containing the white cells and platelets is removed and mixed, and a 0.2ml aliquot is added to 0.8ml of white blood cell (WBC) diluting fluid and a white cell count is performed. 10ml of the leucocyte containing fluid is centrifuged at 1000g for 15 minutes, and the supernatant is discarded. 1.3ml of 5% TCA is added to the pellet of white cells and this is thoroughly homogenised. The homogenate is centrifuged and 1.0ml of the supernatant is added to 0.3ml of reagent in a test tube. The tubes are incubated for 4 hours at 37°C, and allowed to cool in an ice bath. 1.5ml of 65% H_2SO_4 is added and the tubes allowed to stand for 30 minutes. The optical density is read

at 520nm against a reagent blank. A graph prepared from ascorbic acid standards of 0.4, 0.8, 1.6, 4.0 and 10µg/ml of 5% TCA treated as above, is prepared. The ascorbic acid content of the supernatant is read from this graph, and is expressed as µg/10⁸ WBC.

Calculation

$$\mu\text{g ascorbic acid/ml supernatant} \times \frac{1.3 \times 10^8}{\text{No of WBC in 1Cml}}$$

$$= \mu\text{g ascorbic acid}/10^8 \text{ WBC}$$

Protein Estimation

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The method of Lowry et al was used

Solutions

- A: 2% Na_2CO_3 in 0.1N NaOH
- B: 2% Sodium tartrate
- C: 1% Copper sulphate
- D: Folin-Ciocalteu reagent diluted 1:1 with water.

To 100ml of solution A add 1ml of solution B and 1ml of solution C, this reagent must be renewed daily. To 5ml of this working solution add 1ml of a diluted protein sample containing 10-100 μg of protein/ml. Mix well and allow to stand for 10 minutes at room temperature. Add 0.5ml of solution D and mix immediately. After 30 minutes read the optical density at 750nm against a water blank treated in the same way.

Bovine serum albumin (fraction V) standards diluted to contain 25, 50, 75 and 100 μg protein/ml are treated as above, and a standard graph of optical density against protein concentration is constructed (Fig 10). The concentration of the protein samples can be read of the graph and multiplied by their dilution to obtain the protein concentration of the original sample.

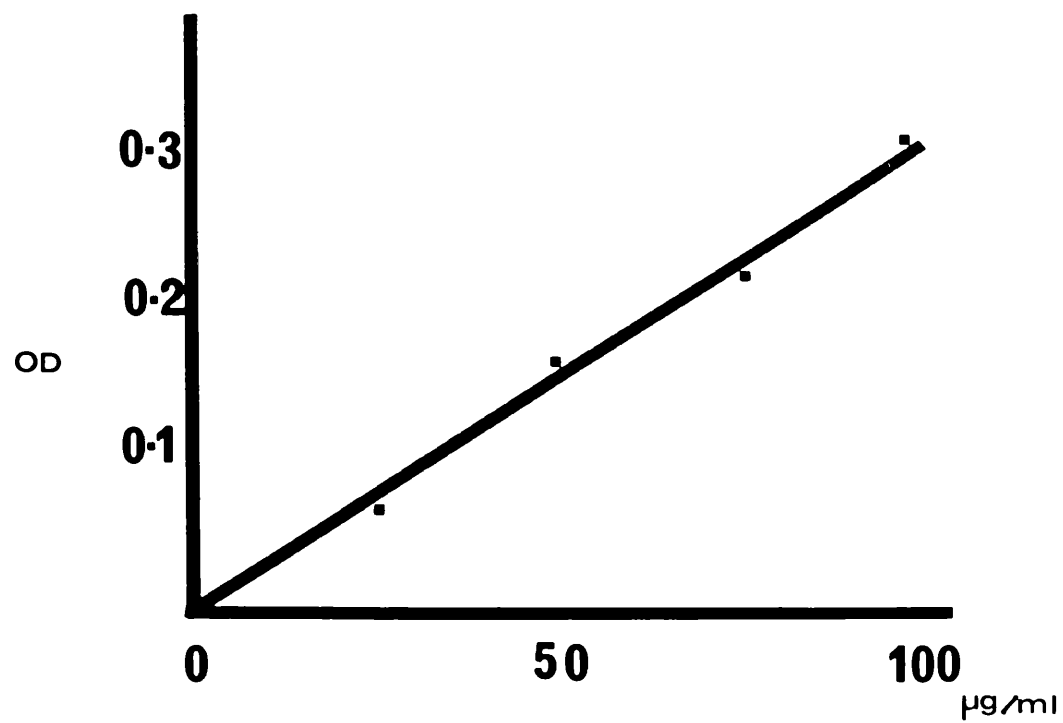


FIG. 10 Protein standard graph

Statistical Methods

Results are calculated and expressed as means \pm standard deviation (S.D.), the standard deviation being calculated from the expression:

$$S.D. = \sqrt{\frac{\sum \{X - \bar{X}\}^2}{n-1}}$$

where X is any of the values measured, \bar{X} is the mean value, and n the number of observations.

The significance of results was calculated by Students t test using the formula:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{s \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where s is an estimate of the combined standard deviation of both groups calculated from:

$$s^2 = \frac{\sum \{X - \bar{X}_1\}^2 + \sum \{X - \bar{X}_2\}^2}{n_1 + n_2 - 2}$$

\bar{X}_1 and \bar{X}_2 are the means of both groups and n_1 and n_2 the number of observations in each group.

In some of the human studies where a normal distribution was not assumed, the Mann-Whitney U test was used.

$$U = n_1 n_2 + \frac{n_1 (n_1 + 1)}{2} - R_1$$

or equivalently,

$$U = n_1 n_2 + \frac{n_2 (n_2 + 1)}{2} - R_2$$

where R_1 = sum of the ranks assigned to group whose sample size is n_1

R_2 = sum of the ranks assigned to group whose sample size is n_2

The method of least-squares was used to obtain the regression coefficient of lines, and from this the correlation coefficient r .

With n pairs of associated observations represented by (x, y) ,
the true regression line for the regression of y on x is:

$$y = a + \beta x$$

the estimate of the true regression coefficient β is:

$$b = \frac{\sum \{x - \bar{x}\} \{y - \bar{y}\}}{\sum \{x - \bar{x}\}^2}$$

Similarly, the true regression line for the regression of x on y is:

$$x = a' + \beta' y$$

and the estimate of the true regression coefficient β' is:

$$b' = \frac{\sum \{x - \bar{x}\} \{y - \bar{y}\}}{\sum \{y - \bar{y}\}^2}$$

The correlation coefficient $r = \sqrt{bb'}$

CHAPTER 1.

ANIMAL EXPERIMENTS.

SECTION 1.

PRELIMINARY CONSIDERATIONS.

Animal of Choice

In the field of alcohol research animal experimental models must be made, as far as possible, applicable to man, if an understanding and possible solution of an entirely human problem is to be achieved. Any attempt to extrapolate results obtained from ethanol metabolism experiments on animals, and apply them to man, has inherent difficulties due to species differences. These differences can be minimised by choosing an animal that is as similar as possible to man, with respect to tolerance and metabolic handling of ethanol.

The rat has been extensively used for experimental studies on ethanol metabolism, but there are disadvantages in its use. The tolerance of the rat to ethanol is much greater than that of man, the lethal blood ethanol concentration in the rat⁴⁵ being 900mg/100ml, compared with 500mg/100ml in man⁴⁷. The rate of ethanol metabolism in the rat,¹⁰¹ judged by blood ethanol clearance rates, is 45mg ethanol/100ml blood/hr, which is much greater than that of man¹⁰¹ (15mg ethanol/100ml blood/hr).

Although the guinea pig has been less widely studied than the rat, in many ways it is a better choice of animal for studies on ethanol metabolism. The lethal blood ethanol concentration of the guinea pig is similar to that of man; an oral dose of 4g ethanol/Kg body wt results in a lethal blood ethanol concentration of 500mg/100ml in both man and the guinea pig⁴⁵. In contrast, the rat requires an oral dose of approximately 9g ethanol/Kg body wt to reach its lethal blood ethanol concentration of 900mg/100ml⁴⁵. Jervis¹⁰² found the blood ethanol clearance rate of guinea pigs to be 33mg/100ml/hr. A further important similarity between the guinea pig and man is that both species are

unable to synthesise ascorbic acid.

Because of the above similarities between the guinea pig and man, with respect to tolerance and metabolic handling of ethanol, and the inability of both species to synthesise ascorbic acid, the guinea pig was the animal of choice for the present studies.

Length of time on ascorbic acid deficient diet.

Guinea pigs fed an ascorbic acid deficient diet for 12-16 days show no obvious signs of scurvy, but have increased plasma half lives ($t_{1/2}$) of a number of drugs⁷⁶. This is due to decreased microsomal drug metabolizing enzyme activities and decreased concentrations of microsomal electron transport components^{77,78,103}. The ascorbic acid deficient guinea pig is therefore a useful animal model for studying the effects of decreases in the activity of the microsomal drug metabolizing system on ethanol metabolism. A preliminary experiment was therefore carried out to establish a dietary regimen, which would give similar results to those of the previous studies outlined above. The serum $t_{1/2}$ of diphenylhydantoin (DPH) was used to assess the extent of the decrease in microsomal drug metabolism.

Materials and Methods.

Twelve male albino Hartley strain guinea pigs (A. Tuck and Son Ltd., Rayleigh, Essex) weighing 200-250g were fed an ascorbic acid deficient diet for 14 days. The composition of the diet is given in the Methods section. This strain and weight of guinea pig was used in all the following animal experiments. Six guinea pigs were given a daily supplement of 50 mg ascorbic acid, dissolved in 1.0ml water, by gastric intubation. These ascorbic acid supplemented animals were the normal controls for the ascorbic acid deficient group.

Liver ascorbic acid concentration and serum $t_{\frac{1}{2}}$ of DPH were measured in each animal. The details of these estimations are given in the Methods section. The body weights of the animals were also noted during the experiment.

Results.

In fig 11, the mean body weights of the two groups of animals are plotted against time on the ascorbic acid deficient diet. There was no significant difference in the body weights of the two groups of animals over the period of the experiment. The concentration of liver ascorbic acid and the serum $t_{\frac{1}{2}}$ of DPH are given in table 1, for both groups of animals. The liver ascorbic acid concentration of the ascorbic acid deficient group was significantly lower ($P < 0.001$) than that of the normal animals, and this represented a reduction to 28 per cent of the concentration found in the normal animals. The serum $t_{\frac{1}{2}}$ of DPH was significantly increased ($P < 0.005$) from 5.5 ± 0.75 hrs in the normal animals to 7.5 ± 0.92 hrs in the ascorbic acid deficient animals. This represents an increase of approximately 36 per cent in the serum $t_{\frac{1}{2}}$ of DPH in the ascorbic acid deficient animals compared with that found in the normal animals.

There were no signs of scurvy in the ascorbic acid deficient animals after 14 days on the ascorbic acid deficient diet.

Discussion.

The increase in serum $t_{\frac{1}{2}}$ of DPH found in the present study is in agreement with the results obtained by Axelrod et al⁷⁶, who demonstrated increases in plasma $t_{\frac{1}{2}}$ of acetanilide, aniline and antipyrine in guinea pigs of similar weight (260-280g), maintained on an ascorbic acid deficient diet for a similar time period (16 days).

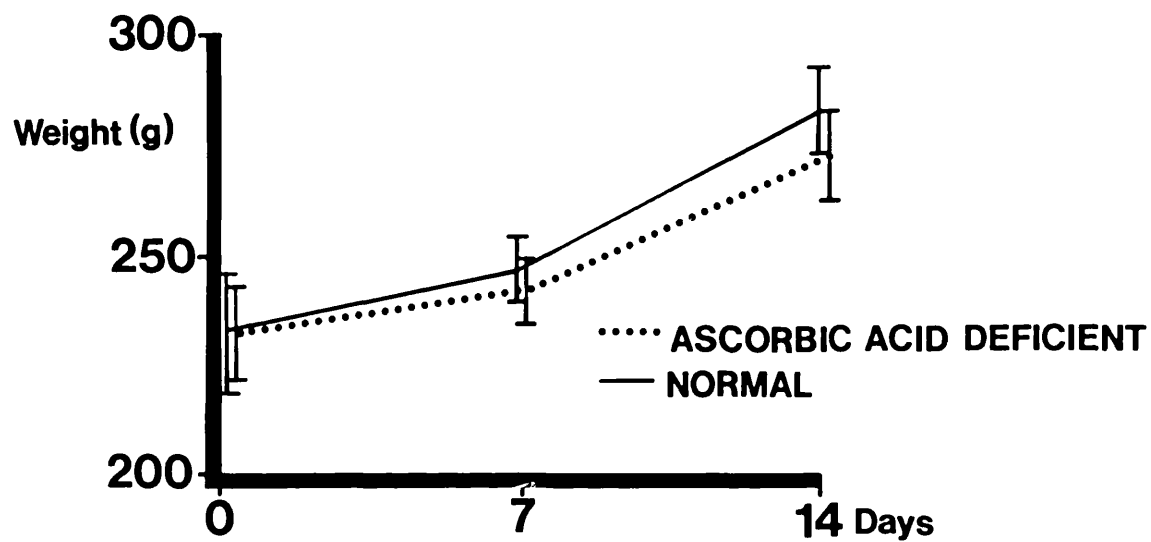


FIG. II

Body weight changes with time in normal and ascorbic acid deficient guinea pigs.

Table 1. Liver ascorbic acid concentration and serum half life ($t_{1/2}$) of diphenylhydantoin in normal and ascorbic acid deficient guinea pigs*.

	Liver ascorbic acid ($\mu\text{g/g}$ wet wt)	Diphenylhydantoin $t_{1/2}$ (hrs)
Normal	170 ± 22	5.5 ± 0.75
Ascorbic acid deficient	48 ± 6	7.5 ± 0.92
Significance P <	0.001	0.005

* Each figure represents the mean of six animals \pm S.D.

Several species, including man¹⁰⁵ and the rat¹⁰⁴, metabolize DPH mainly by ring hydroxylation in the para position to 5-p-OH-phenyl-5-phenylhydantoin. The serum $t_{1/2}$ of 5.5 hrs found in the normal guinea pigs in the present study compares with that of 10 hrs found in man¹⁰⁶. The increase in serum $t_{1/2}$ of DPH in the ascorbic acid deficient guinea pigs probably reflects a decrease in the activities of the microsomal hydroxylase enzymes responsible for its ring hydroxylation, as has been previously shown for other drugs by several workers^{77,78,103}.

Kuether et al¹⁰⁷ found the concentration of liver ascorbic acid in guinea pigs fed a normal chow diet supplemented daily with cabbage to be 260 $\mu\text{g/g}$ wet wt, which is the same as that found in the livers of species like the rat and mouse¹⁰⁸ which are able to synthesise ascorbic acid. This concentration must therefore represent total tissue saturation of ascorbic acid. The liver ascorbic acid concentration of 170 $\mu\text{g/g}$ wet wt obtained in the present study compares favourably with the concentration of 190 $\mu\text{g/g}$ wet wt found by Zannoni et al¹⁰⁹ in animals of the same weight receiving the same daily supplement of ascorbic acid. It is difficult to achieve complete saturation of ascorbic acid in guinea pig liver with daily oral supplements. Sato and Zannoni¹¹⁰ found that even 75 mg/day of ascorbic acid given orally was not sufficient to saturate the livers of weanling guinea pigs. The problem of increasing the gastrointestinal absorption of ascorbic acid can be overcome by giving intramuscular injections of the vitamin, but this in turn can lead to stress effects and can actually lower the tissue ascorbic acid concentration⁸¹. Supplementing the normal animals' diet with cabbage leads to tissue saturation, but this would introduce other dietary differences between the normal and ascorbic acid deficient animals. It was therefore accepted that the guinea pigs used in the present study were not completely saturated with ascorbic acid.

As the 14 day dietary regimen used was sufficient to bring about

changes in microsomal drug metabolizing enzyme activities, as judged by the increase in serum $t_{\frac{1}{2}}$ of DPH, it was decided to adhere to the above dietary conditions for all further experiments involving ascorbic acid deficient guinea pigs.

SECTION 2

REDOX STATE CHANGES WITH ETHANOL.

The redox state of the various subcellular compartments of the liver is shifted to a more reduced level during ethanol metabolism, due to the increased production of NADH by ADH and other NAD^+ dependent dehydrogenases involved in ethanol metabolism⁵⁵. This shift in hepatic redox state can influence both the metabolism of ethanol¹¹¹, and the biochemical changes resulting from its metabolism^{112, 113}.

One example of the latter effect is ethanol induced impairment of the TCA cycle flux^{112, 113} which is thought to be due to the increase in the cytoplasmic and intramitochondrial NADH/NAD^+ ratios. Lindros¹¹² using perfused liver from normal, hypothyroid and hyperthyroid rats, showed that there was a good correlation between the extent of the redox state shift with ethanol, and the degree of impairment of the TCA cycle flux.

The dissociation of the ADH-NADH complex is considered the slowest step in the ADH reaction⁸, consequently the redox state, and in particular the concentration of free NADH in the cytoplasm, will affect this dissociation and contribute to the control of the ADH reaction. Decreases in the activity of ADH can also affect ethanol oxidation, as Papenberg¹¹⁴ has shown that the administration of pyrazole, which inhibits ADH activity, lowers the rate of elimination of ethanol in perfused rat liver. In animals with normal or raised ADH activities however, it is the hepatic redox state that is the major regulatory mechanism of ethanol oxidation¹¹⁵.

Previous studies on redox state changes after ethanol administration have been mainly acute studies carried out on rats^{55, 111-113, 115}. Only two studies have been carried out on chronic ethanol treated rats^{116, 117}. As far as can be established, no previous work has been carried out on

the in vivo effect of ethanol on the hepatic redox state of the guinea pig. It was therefore decided to investigate the effects of both acute and chronic ethanol administration on the hepatic redox state of normal and ascorbic acid deficient guinea pigs. Starvation, which alters the hepatic redox state of rats⁸⁷, was also studied in guinea pigs.

Materials and Methods

Redox state changes after ethanol administration to starved guinea pigs.

Twelve male albino guinea pigs, weighing 200-250g, were starved for 72hr. During this time they were allowed access to water and received 50mg ascorbic acid daily by gastric intubation. At the end of this 72 hr period, six animals were injected i.p. with 1.5g ethanol/Kg body wt as a 30% (W/V) solution in isotonic saline. The remaining six animals acted as controls and received the same volume of isotonic saline. After 30 min the animals were killed by cervical dislocation and their livers rapidly removed and freeze clamped. Metabolites were extracted and measured as described previously in the Methods section.

Redox state changes after ethanol administration to chronic ethanol treated normal and ascorbic acid deficient guinea pigs.

Male albino guinea pigs, weighing 200-250g, were fed the ascorbic acid deficient diet, described previously in the Methods section, for 14 days. The ethanol pretreated groups were intubated daily with 2.5g ethanol/Kg body wt as a 50% (W/V) solution in water. Control groups received isocaloric quantities of glucose. Half the ethanol and glucose groups received a daily supplement of 50mg ascorbic acid dissolved in their respective intubation solutions. These animals were the normal controls for the ascorbic acid deficient groups. The animals were

fasted overnight (18 hr) on the 14th day and injected i.p with 1.5g ethanol/Kg body wt as a 30% (W/V) solution in isotonic saline. Control animals received the same volume of isotonic saline. After 30 min the animals were killed by cervical dislocation and their livers rapidly removed and freeze clamped. Metabolites were extracted and measured as previously described in the Methods section.

Results.

The cytoplasmic redox state of the starved animals, expressed as the calculated free NADH/NAD^+ ratio (table 2) is in a more reduced state than that of the normal and ascorbic acid deficient animals fasted 18 hr (table 3). Conversely, the intramitochondrial redox state of the starved animals (table 2) is in a more oxidised state than that of the normal and ascorbic acid deficient animals (table 4). These differences in redox states can be related to differences in the concentration of the metabolites used to calculate them. The lactate and pyruvate concentrations of the starved animals are lower than those of the normal and ascorbic acid deficient animals, whereas the 3-OH-butyrate and acetoacetate concentrations are much higher in the starved animals. After ethanol injection the cytoplasmic and intramitochondrial redox states of the starved animals are similar to those of the ethanol injected normal and ascorbic acid deficient animals. The weight of the starved animals was reduced by 28% at the end of the 72 hr period of starvation.

The well documented cytoplasmic redox state shift after ethanol administration is demonstrated in table 3, for all the normal and ascorbic acid deficient groups injected with ethanol, compared with their saline injected controls. There is a significant ($P < 0.02$)

Table 2. Cytoplasmic and intramitochondrial redox state changes with ethanol in starved guinea pigs.

	Starved	Starved + ethanol
Lactate	535 \pm 67	1036 \pm 123
Pyruvate	21 \pm 3	26 \pm 4
Lactate/pyruvate	26.8 \pm 1.7	38.8 \pm 2.1
Cytoplasmic * free NADH/NAD ⁺ X 10 ⁴	30.1 \pm 1.9	44.3 \pm 2.6
3-Hydroxybutyrate	149 \pm 10	252 \pm 22
Acetoacetate	259 \pm 18	190 \pm 15
3-Hydroxybutyrate/acetoacetate	0.59 \pm 0.12	1.34 \pm 0.15
Intramitochondrial ** free NADH/NAD ⁺ X 10 ²	2.89 \pm 0.53	6.52 \pm 0.71

* Calculated from the lactate/pyruvate ratio of each animal using the value of 1.11×10^{-4} for the equilibrium constant of lactate dehydrogenase.⁸⁷

** Calculated from the 3-hydroxybutyrate/acetoacetate ratio of each animal using the value of 4.93×10^{-2} for the equilibrium constant of 3-hydroxybutyrate dehydrogenase.⁸⁷

Table 3. Cytoplasmic redox state changes with ethanol in normal and ascorbic acid deficient (scorbutic) guinea pigs*.

Groups	Injection	Lactate	Pyruvate	Lactate/pyruvate	Free $\frac{\text{NADH}}{\text{NAD}^+} \times 10^4$
Scorbutic	Saline	780 \pm 150	39 \pm 5	21.4 \pm 2.5	23.8 \pm 2.8
Scorbutic	Ethanol	1106 \pm 163	28 \pm 6	** 38.9 \pm 2.6	43.2 \pm 2.9
Scorbutic + chronic ethanol	Saline	793 \pm 155	38 \pm 5	19.6 \pm 2.7	20.7 \pm 3.0
Scorbutic + chronic ethanol	Ethanol	1024 \pm 159	26 \pm 4	** 34.4 \pm 3.0	38.2 \pm 3.2
Normal	Saline	750 \pm 149	33 \pm 5	21.1 \pm 2.4	24.7 \pm 2.7
Normal	Ethanol	1044 \pm 161	24. \pm 4	* 40.1 \pm 2.5	44.5 \pm 2.3
Normal + chronic ethanol	Saline	712 \pm 140	31 \pm 5	21.4 \pm 1.9	23.8 \pm 2.2
Normal + chronic ethanol	Ethanol	1012 \pm 158	25. \pm 5	* 36.1 \pm 2.8	40.2 \pm 3.1

* Metabolite concentrations are nmoles/g wet wt liver. Each figure is the mean \pm S.D. of six animals.

** and * differ by $P < 0.02$.

Table 4. Intramitochondrial redox state changes with ethanol in normal and ascorbic acid deficient (scorbutic) guinea pigs*.

Groups	Injection	3-Hydroxybutyrate	Acetoacetate	3-Hydroxybutyrate/ acetoacetate	Free $\frac{\text{NADH}}{\text{NAD}^+} \times 10^2$
Scorbutic	Saline	67 \pm 12	59 \pm 9	1.10 \pm 0.12	5.35 \pm 0.61
Scorbutic	Ethanol	78 \pm 14	55 \pm 6	**1.42 \pm 0.11	6.95 \pm 0.63
Scorbutic + chronic ethanol	Saline	66 \pm 10	59 \pm 8	1.08 \pm 0.12	5.28 \pm 0.60
Scorbutic + chronic ethanol	Ethanol	70 \pm 11	58 \pm 7	**1.20 \pm 0.12	5.87 \pm 0.61
Normal	Saline	68 \pm 10	58 \pm 8	1.11 \pm 0.10	5.51 \pm 0.57
Normal	Ethanol	77 \pm 14	54 \pm 7	* 1.43 \pm 0.12	6.98 \pm 0.68
Normal + chronic ethanol	Saline	67 \pm 11	59 \pm 8	1.11 \pm 0.12	5.41 \pm 0.60
Normal + chronic ethanol	Ethanol	71 \pm 12	58 \pm 6	* 1.21 \pm 0.11	6.02 \pm 0.53

** and * differ by $P < 0.01$

* Metabolite concentrations are nmoles/g wet wt liver. Each figure is the mean \pm S.D. of six animals.

decrease in the extent of the redox state shift after ethanol in both the normal and ascorbic acid deficient chronic ethanol treated guinea pigs, compared with their respective unpretreated controls. This is also apparent in table 4 for the intramitochondrial redox state changes after ethanol administration. Again the chronic ethanol treated animals show a significant decrease ($P < 0.01$) in the extent of their redox state shift after ethanol administration, compared with their unpretreated controls. No statistical difference can be seen between the redox states of the ascorbic acid deficient groups and their normal controls.

Discussion.

The possibility of starvation affecting the ascorbic acid deficient guinea pigs can be eliminated, as their metabolite concentrations are similar to those of the normal animals, and both groups have very different metabolite concentrations from those of the starved animals. Williamson et al⁸⁷ have shown that 48 hr starvation in the rat produces a parallel shift in the cytoplasmic and intramitochondrial redox state to a more reduced state. The 72 hr starved guinea pigs used in the present work showed similar changes to those demonstrated by Williamson et al⁸⁷, in that their cytoplasmic redox state was in a more reduced state than that of the 18 hr fasted guinea pigs, but their intramitochondrial redox state was shifted to a more oxidised state. It is possible that the length of time of starvation has produced a severe state of ketosis, as shown by the large increases in the ketone bodies acetoacetate and 3-OH-butyrate, and that this is affecting the equilibrium of the intramitochondrial redox state pairs. This may explain the non-parallel changes in redox

state in the cytoplasmic and intramitochondrial compartments of the starved guinea pig. After ethanol administration there is a shift to a more reduced redox state in both compartments, which indicates that the starved guinea pig can still respond metabolically to ethanol, as Smith and Newman have shown for starved rats⁵⁵.

Lindros and Hillbom¹¹⁸ have shown that there are parallel changes to a more reduced state, in the lactate/pyruvate and 3-OH-butyrate/acetoacetate ratios after ethanol administration to normal fed rats. This has also been demonstrated in the present work for the normal and ascorbic acid deficient groups injected with ethanol.

The decrease in the extent of the redox state shift after ethanol injection in the chronic ethanol treated guinea pigs, compared with that found in their respective unpretreated controls, is in agreement with results obtained by Domschke et al¹¹⁷. These workers demonstrated an attenuation in the redox state shift after acute ethanol administration to chronic ethanol treated rats, compared with the redox state shift after acute ethanol administration to control animals, which had previously been given isocaloric amounts of carbohydrate. The chronic ethanol treated rats in this study received 13-15g ethanol/Kg body wt/day as part of a liquid diet over 25 days. This is a much larger dose given over a longer period of time, than that producing similar results in the guinea pigs of the present study.

The results of Gordon¹¹⁶, who also studied chronic ethanol treatment in rats receiving ethanol as part of a liquid diet, are difficult to relate to those of Domschke et al¹¹⁷ and the present study, as no acute ethanol treated control animals were studied. Gordon¹¹⁶ demonstrated that the 3-OH-butyrate/acetoacetate ratio of chronic

ethanol treated rats was in a more reduced state than that of control animals who had received no ethanol. There was no increase in the lactate/pyruvate ratio of the chronic ethanol treated rats. As details of the blood ethanol concentration and amount of ethanol ingested prior to sacrifice were not given for these animals, it is difficult to assess the actual contribution of ethanol intake to these results.

In the present study, the decrease in the extent of the redox state shift with ethanol in the chronic ethanol treated guinea pig, cannot be due to differences in blood ethanol concentration between the acute and chronic ethanol treated guinea pigs. The theoretical zero time concentration of ethanol in the blood is the same for both groups (table 7).

The decrease in the extent of the redox state shift after ethanol administration to the chronic ethanol treated guinea pig may represent metabolic adaptation to ethanol. Rawat and Kuriyama⁵³ have previously shown that chronic ethanol treated mice have an increased mitochondrial permeability to NADH. Videla et al³¹ have also demonstrated that chronic ethanol treatment in rats results in a faster mitochondrial oxygen consumption, which suggests that these mitochondria can reoxidise NADH at a faster rate. It is uncertain which adaptive mechanism may be operating in the chronic ethanol treated guinea pigs of the present study.

Hillbom¹¹⁵ has previously demonstrated the importance of the redox state in the regulation of ethanol elimination in vivo. An increase in ethanol elimination of 20 mg/kg body wt/hr was obtained in rats treated with promethazine, which decreased the extent of the redox state shift with ethanol by 50 per cent of that of untreated control animals. The 10 per cent decrease in the extent of the redox state shift after ethanol administration, found in the chronic ethanol treated guinea pigs in the present work, is probably too small to affect the rate of ethanol

elimination. . This is substantiated in table 7, as there is no difference in the rate of ethanol elimination from the blood between the chronic ethanol treated guinea pigs and their untreated controls.

SECTION 3.

Ethanol Metabolism in the Ascorbic
Acid Deficient Guinea Pig.

Possible in vitro mechanisms of the microsomal ethanol oxidizing system (MEOS) have been described previously. MEOS has been shown in vitro to be similar to the hepatic microsomal drug metabolizing system³⁴, as it can oxidize ethanol to acetaldehyde in the presence of NADPH and O_2 . The nature of MEOS activity has been widely studied using inhibitors to differentiate between the various possible enzyme reactions involved^{39,40,42,119}. Unfortunately the various enzyme inhibitors used are not entirely specific, and some doubt as to the exact mechanism of MEOS activity still remains.

The in vivo significance of MEOS has also been widely studied using inhibitors of the microsomal drug metabolizing system, in an attempt to establish the extent of microsomal involvement in ethanol metabolism. These agents are again not entirely specific and results obtained are inconclusive. The injection of SKF 525-A, which is a microsomal enzyme inhibitor, had no effect on the rate of ethanol clearance from the blood of rats or the whole body of mice¹²⁰, indicating that ethanol is not metabolized by microsomal enzymes to any significant extent. Unfortunately SKF 525-A also caused a delay in the absorption of ethanol in the rat¹²⁰, and results obtained with this compound are therefore questionable.

The guinea pig is a useful animal model for studies on the significance of MEOS in vivo, as the plasma half-life of a number of drugs is increased in the ascorbic acid deficient animal⁷⁶. This is due to decreased microsomal drug metabolizing enzyme activities, and decreased amounts of microsomal electron transport components^{78,103,109}. Phenobarbitone pretreatment returns these decreased microsomal enzyme activities to normal, indicating that the microsomal enzyme protein

synthesising mechanism is operable in ascorbic acid deficient guinea pigs¹⁰⁹.

In the present study non-specific effects introduced by microsomal enzyme inhibitors were avoided by the use of the ascorbic acid deficient guinea pig as an animal model. Experiments were carried out to assess the effect of ascorbic acid deficiency and chronic ethanol treatment on ADH activity. The effect of decreased microsomal electron transport components and decreased microsomal enzyme activities on the rate of ethanol elimination in vivo, and the ability of ethanol to induce microsomal enzyme activities was also investigated.

METHODS

Animal Treatment

Male albino guinea pigs, weighing 200-250g were fed the ascorbic acid deficient diet described previously, for 14 days. The ethanol pretreated groups were intubated daily with 2.5g ethanol/Kg body weight as a 50% (W/V) solution. Control groups received isocaloric quantities of glucose. Half the ethanol and glucose groups received a daily supplement of 50mg of ascorbic acid dissolved in their respective intubation solutions. These animals were the normal controls for the ascorbic acid deficient groups.

Ethanol metabolism in vivo.

The animals were fasted overnight (18 hr) on the 14th day and injected i.p. with 1.5g ethanol/Kg body weight as a 30% (W/V) solution in isotonic saline. Blood samples were removed from the ear vein at half-hour intervals for 3 hr, and blood ethanol concentrations and rates of ethanol metabolism were measured as described previously.

Preparation of guinea pig liver microsomes

Animals were treated as above for 14 days but received no ethanol for 24 hr prior to sacrifice. Microsomes were prepared as described previously.

Determinations.

Aniline hydroxylase, cytochrome P-450, cytochrome b_5 , MEOS, NADPH oxidase, catalase, ADH, liver ascorbic acid and protein were measured as described previously.

Results.

No difference in terminal body weight, liver weight, liver/body weight or ADH activity was found between any of the groups of animals studied (Table 5). The liver ascorbic acid concentration was decreased to 30 per cent of normal values in both the ascorbic acid deficient (scurbutic), and chronic ethanol treated ascorbic acid deficient groups. This did not produce any changes in the other parameters measured in Table 5.

Microsomal protein content, aniline hydroxylase and NADPH oxidase activities were significantly decreased in the ascorbic acid deficient groups, compared with their normal controls, although catalase and MEOS activities were unchanged (Table 6). Cytochrome P-450 concentration was significantly decreased in the ascorbic acid deficient animals, but no change in the concentration of cytochrome b_5 was noted. No evidence of microsomal enzyme induction was found in any of the ethanol pretreated groups.

Pretreatment of guinea pigs with 2.5g ethanol/Kg body weight/day for 14 days had no effect on the rate of clearance of ethanol from the blood, or calculated rate of ethanol metabolism (Table 7). Ascorbic acid deficiency also had no effect on the rate of ethanol metabolism

Table 5. Effect of chronic ethanol treatment and ascorbic acid deficiency on body weight, liver weight, ADH activity and liver ascorbic acid content of guinea pigs*.

	Normal	Scorbutic	Normal + chronic ethanol	Scorbutic + chronic ethanol
Terminal body wt (g)	286 ± 17	273 ± 32	266 ± 29	269 ± 33
Liver wt (g)	11.0 ± 1.0	10.2 ± 1.4	10.0 ± 0.8	10.5 ± 1.3
Liver wt/body wt	0.038 ± 0.002	0.037 ± 0.003	0.037 ± 0.003	0.039 ± 0.003
ADH activity (U/g protein)	9.6 ± 1.5	9.9 ± 1.4	9.8 ± 0.9	10.4 ± 1.7
Liver ascorbic acid (µg/g wet wt)	164 ± 23	46 ± 7	159 ± 19	42 ± 5

* Each figure represents the mean ± S.D. of six animals.

Table 6. Effect of chronic ethanol treatment and ascorbic acid deficiency on microsomal enzyme activities, electron transport components and protein content.

	Normal	Scorbutic	Normal + chronic ethanol	Scorbutic + chronic ethanol
Aniline hydroxylase (nmoles p-aminophenol produced/mg microsomal protein/hr)	58 ± 7	45 ± 5 **	61 ± 8	46 ± 7 **
NADPH oxidase (nmoles NADPH oxidised/mg microsomal protein/ min)	8.3 ± 1.3	6.0 ± 1.1 *	8.2 ± 1.4	5.9 ± 1.0 **
Catalase (μmoles O ₂ evolved/ mg microsomal protein/min)	9.2 ± 1.2	9.5 ± 1.5	9.3 ± 1.8	9.1 ± 1.9
MEOS (nmoles acetaldehyde produced/mg microsomal protein/ min)	1.60 ± 0.22	1.60 ± 0.28	1.61 ± 0.26	1.60 ± 0.20
Cytochrome P-450 (nmoles/mg microsomal protein)	0.934 ± 0.073	0.709 ± 0.073*	0.963 ± 0.092	0.709 ± 0.086*
Cytochrome b ₅ (ΔE _{423-500nm} /mg microsomal protein)	0.073 ± 0.009	0.078 ± 0.008	0.070 ± 0.006	0.070 ± 0.009
Microsomal protein content (mg/g wet wt liver)	9.90 ± 0.50	8.78 ± 0.68 **	9.65 ± 0.71	8.31 ± 0.77 *

Significance: ** P < 0.01 * P < 0.02 * P < 0.001 with respect to normal controls.

Table 7. Ethanol metabolism in vivo in chronic ethanol treated normal and ascorbic acid deficient guinea pigs*.

	C _O (mg ethanol/100ml blood)	r	Disappearance of Ethanol from blood (mg/100ml/hr)	Ethanol metabolised (mg/Kg body wt./hr)
Normal	153.3 ± 11.1	0.970 ± 0.053	29.3 ± 2.7	230.4 ± 13.2
Scorbutic	152.8 ± 10.3	0.984 ± 0.071	28.1 ± 2.6	274.5 ± 12.9
Normal + chronic ethanol	154.8 ± 7.4	0.970 ± 0.025	28.6 ± 2.2	273.8 ± 11.5
Scorbutic + chronic ethanol	152.0 ± 8.3	0.986 ± 0.063	28.4 ± 2.8	276.6 ± 12.4

* each figure represents the mean of six animals ± S.D.

in vivo, in guinea pigs fed an ascorbic acid deficient diet for 14 days.

Discussion

As far as can be established, the only previous study on ethanol metabolism in ascorbic acid deficient guinea pigs is that of Jervis¹⁰², who showed that ascorbic acid deficient guinea pigs had a decreased rate of clearance of ethanol from their blood. In this study only one ascorbic acid deficient and one control animal was studied and no statistical interpretation of the results was therefore possible. The ascorbic acid deficient animal was studied after 4 weeks on an ascorbic acid deficient diet, which resulted in loss of weight and haemorrhage of joints, and it is likely that starvation caused the decrease in the rate of ethanol clearance from the blood of this animal. Smith and Newman⁵⁵ have demonstrated that starvation leads to impaired ethanol metabolism in the rat. A further objection to the methods used in the study of Jervis¹⁰², is that blood samples were obtained by multiple cardiac puncture which can lead to pericardial haemorrhage and affect the results obtained. Unfortunately Jervis¹⁰² did not measure any of the enzymes involved in ethanol metabolism in these guinea pigs.

In the present study the lack of effect of chronic ethanol treatment on the activity of guinea pig ADH is in agreement with the results of other workers, who failed to show any induction of ADH activity in chronic ethanol treated rats.^{40,121,122.}

The decrease in aniline hydroxylase activity in the ascorbic acid deficient guinea pigs is in agreement with results of Kato¹⁰³ and Wade et al,¹²³ who studied guinea pigs fed an ascorbic acid deficient diet for 12 days. Wade et al¹²³ also showed a decrease in the concentration of cytochrome P-450 and microsomal protein, but no change in the

concentration of cytochrome b_5 in these animals, which is in agreement with results obtained in the present study.

Previous workers have demonstrated that chronic ethanol treatment of rats leads to induction of microsomal enzyme activities^{31,124} and increased concentrations of cytochrome P-450¹²². These rats were fed 12-14g ethanol/Kg body weight/day in a semi-liquid diet for 14 days,^{31,122} which also resulted in an increased rate of ethanol elimination from the blood. Induction of microsomal systems depends on the quantity of ethanol and its mode of administration. In the present study 2.5g ethanol/Kg body weight was given as a single daily oral dose for 14 days, and this could not be raised as the LD_{50} for a single oral dose of ethanol is 4g/Kg body weight in guinea pigs⁴⁵.

In contrast to the present study, several workers have previously shown that NADPH oxidase activity is increased in chronic ethanol treated rats,^{30,31,39} again the amount of ethanol and its mode of administration may contribute to the difference in these results. MEOS activity is also increased after chronic ethanol treatment in rats,^{30,34,122} while catalase activity is unaltered³¹. It has been suggested by Carter and Isselbacher³⁰ that the increase in MEOS activity is due to the increase in NADPH oxidase activity in chronic ethanol treated rats. These authors consider MEOS activity to be a product of NADPH oxidase and catalase activity.

If MEOS activity is a product of NADPH oxidase and catalase activity, the decrease in NADPH oxidase activity found in the ascorbic acid deficient guinea pig should result in a decrease in MEOS activity, as NADPH oxidase and its production of H_2O_2 is considered the rate limiting step in this system³¹. It is possible that NADPH oxidase

activity is not sufficiently reduced in the ascorbic acid deficient guinea pig to bring about a reduction in MEOS activity.

From the results of the present study it is not possible to establish the precise involvement of NADPH oxidase and catalase in MEOS activity, but it is obvious that MEOS activity is not related to the activity of the microsomal drug metabolizing system. The concentration of cytochrome P-450 was decreased in the ascorbic acid deficient guinea pig, resulting in a decrease in the activity of aniline hydroxylase, a 'true' microsomal drug metabolizing enzyme, whereas MEOS activity was unchanged and did not appear to be dependent on the concentration of cytochrome P-450.

This is in agreement with more recent in vitro studies of Vatsis et al¹²⁵, who showed that preincubation of mouse liver microsomes with NADPH resulted in peroxidation of unsaturated fatty acids of the microsomal phospholipids. This resulted in a destruction of cytochrome P-450 and decreased activities of aniline hydroxylase, although no change in the activities of MEOS or NADPH oxidase were evident. These authors also concluded that cytochrome P-450 was not involved in MEOS activity.

In conclusion, no evidence has been found for microsomal involvement in MEOS activity in vitro, or ethanol metabolism in vivo; as a reduction in microsomal drug metabolizing enzyme activities and decreased cytochrome P-450 concentrations, had no effect on MEOS activity or ethanol elimination from the blood. Drugs that are substantially metabolized by the microsomal drug metabolizing system have increased plasma half-lives in ascorbic acid deficient guinea pigs,⁷⁶ but no decrease in the rate of elimination of ethanol from the blood was

observed in the ascorbic acid deficient guinea pigs used in the present study. When ADH activity is normal no further systems appear to be required for ethanol metabolism. This is in agreement with previous studies on the significance of MEOS in vivo, except in the present work non-specific effects of inhibitors of microsomal drug metabolizing enzymes have been avoided.

SECTION 4.

ETHANOL METABOLISM IN THE PHENOBARBITONE

TREATED GUINEA PIG.

As the effect of decreased microsomal enzyme activities on the rate of ethanol metabolism was investigated in the previous section, it was of interest to investigate the converse system where these enzymes are increased in activity.

Phenobarbitone is a powerful inducer of the microsomal drug metabolizing system¹²⁶, and has been used previously for studies on the effects of increased microsomal enzyme activities on ethanol metabolism.^{30,38,120,127.}

Unfortunately there are disadvantages in the use of barbiturates for studies on ethanol metabolism, as both phenobarbitone and barbitone have been shown to inhibit ADH and prevent the redox state shift after ethanol administration.^{128,129} The rate of ethanol oxidation in mouse liver slices is also decreased in the presence of barbitone.¹²⁹ Kyogoku et al¹³⁰ have demonstrated that barbiturates can bind to adenine nucleotide derivatives, and it is probable that the ADH reaction is inhibited by the binding of NAD^+ to barbiturate.

Khanna et al¹²⁷ showed that 24 hours after the last dose of phenobarbitone, there is no inhibition of ethanol metabolism in rats. If phenobarbitone is therefore allowed to clear from the body prior to investigations on ethanol metabolism, the effect of increased activities of microsomal drug metabolizing enzymes can be studied, without complications arising from enzyme inhibition.

Methods.

Male albino guinea pigs weighing 200-250g were fed the ascorbic acid deficient diet, described previously, for 14 days. Control animals were intubated daily with 50mg ascorbic acid dissolved in 1ml water, and the ascorbic acid deficient animals were intubated with the same volume of water.

Phenobarbitone (0.5mg/ml) was added to the drinking water of half the deficient and control animals on the morning of day 10 of the dietary period, and was removed on the morning of day 14. The phenobarbitone solution was made up fresh each day and the animals drank between 30 and 50ml daily.

In vivo ethanol metabolism was measured, as described previously, after an overnight fast on the 14th day. Enzyme and ascorbic acid determinations were performed on animals treated as above for 14 days, except that they received no ethanol.

Results.

The phenobarbitone treated animals had significantly increased liver and liver/body weights compared with their untreated controls ($P < 0.001$). ADH activity was unchanged by phenobarbitone (Table 8).

The activity of aniline hydroxylase, and the concentrations of cytochrome P-450 and microsomal protein, were significantly increased after phenobarbitone treatment in both the ascorbic acid deficient (scorbutic) and normal phenobarbitone treated animals (Table 9).

NADPH oxidase, catalase and MEOS activities were unchanged, as was the concentration of cytochrome b_5 . Phenobarbitone pretreatment returned the activity of aniline hydroxylase and the concentration of cytochrome P-450, in the phenobarbitone treated ascorbic acid deficient guinea pig, to near normal values.

In agreement with results obtained in the previous section, significant differences were found between the ascorbic acid deficient and normal animals, with regard to aniline hydroxylase and NADPH oxidase activities, and the concentrations of cytochrome P-450 and microsomal protein.

Phenobarbitone pretreatment had no effect on the rate of ethanol

Table 3. Effect of phenobarbitone pretreatment on body weight, liver weight, ADH activity and liver ascorbic acid content of normal and ascorbic acid deficient guinea pigs.

	Normal	Normal + phenobarbitone	Ascorbic acid deficient	Ascorbic acid deficient + phenobarbitone
Terminal body wt (g)	290 ± 23	283 ± 23	280 ± 25	291 ± 30
Liver wt (g)	12.2 ± 1.2	17.5 ± 2.1 P < 0.001	12.7 ± 1.5	18.7 ± 2.3 P < 0.001
Liver wt/body wt	0.041 ± 0.003	0.058 ± 0.004 P < 0.001	0.044 ± 0.004	0.061 ± 0.005 P < 0.001
ADH activity (U/g protein)	9.7 ± 1.3	10.1 ± 1.5	9.9 ± 1.2	10.3 ± 1.6
Liver ascorbic acid (µg/g wet wt)	176 ± 28	161 ± 23	49 ± 8	42 ± 7

Each figure represents the mean ± S.D. of six animals.

Table 9. Effect of phenobarbitone pretreatment on microsomal enzyme activities, electron transport components, and protein content of normal and ascorbic acid deficient (scorbutic) guinea pigs.

	Normal	Normal + phenobarbitone	Scorbutic	Scorbutic + phenobarbitone
Aniline hydroxylase (nmoles p-aminophenol produced/mg microsomal protein/hr	60 ± 8 P < 0.001	83 ± 7	* 46 ± 5 P < 0.005	61 ± 7*
NADPH oxidase (nmoles NADPH oxidised/mg microsomal protein/min)	8.4 ± 1.2 N.S.	8.7 ± 1.3	** 6.3 ± 1.4 N.S.	6.4 ± 1.3**
Catalase (μmoles O ₂ evolved/ mg microsomal protein/min)	9.5 ± 1.3 N.S.	9.8 ± 1.6	9.3 ± 1.5 N.S.	9.7 ± 1.8
MEOS (nmoles acetaldehyde produced/mg microsomal protein/min)	1.63 ± 0.21 N.S.	1.64 ± 0.26	1.61 ± 0.25 N.S.	1.63 ± 0.28
Cytochrome P-450 (nmoles/ mg microsomal protein)	0.928 ± 0.071 P < 0.001	1.792 ± 0.097	* 0.713 ± 0.073 P < 0.001	1.149 ± 0.089*
Cytochrome b ₅ (ΔE _{423-500nm} /mg microsomal protein)	0.071 ± 0.008 N.S.	0.073 ± 0.009	0.075 ± 0.008 N.S.	0.070 ± 0.006
Microsomal protein content (mg/g wet wt liver)	9.94 ± 0.62 P < 0.001	13.78 ± 0.94	** 8.86 ± 0.72 P < 0.001	11.44 ± 0.83*

Each figure represents the mean ± S.D. of six animals.

Significance: ** P < 0.02 * P < 0.005 * P < 0.001 with respect to normal controls.

Table 10. Rate of clearance of ethanol from the blood of phenobarbitone pretreated normal and ascorbic acid deficient guinea pigs.

	C (mg ethanol/100ml blood)	r	Rate of clearance of ethanol from blood (mg/100ml/hr)	Ethanol metabolized (mg/Kg body wt/hr)
Normal	152.3 ± 8.4	0.987 ± 0.042	30.1 ± 2.8	296.1 ± 15.3
Normal + phenobarbitone	153.8 ± 12.5	0.975 ± 0.067	31.4 ± 2.6	303.3 ± 13.7
Ascorbic acid deficient	152.6 ± 9.7	0.981 ± 0.049	29.8 ± 2.7	292.3 ± 16.9
Ascorbic acid deficient + phenobarbitone	154.2 ± 11.8	0.973 ± 0.054	30.2 ± 2.8	295.5 ± 17.2

clearance from the blood or on the calculated rate of ethanol metabolism, in either the ascorbic acid deficient or normal phenobarbitone pretreated groups (Table 10).

Discussion.

Guinea pig ADH was not affected by phenobarbitone when its activity was measured 24 hours after phenobarbitone administration, which suggests that phenobarbitone had been cleared from the body or was present in insufficient quantities to interfere with the ADH reaction.

The increase in liver/body weight and microsomal protein in the phenobarbitone treated guinea pigs is in agreement with results obtained with phenobarbitone treated rats¹²⁷.

Zannoni et al¹⁰⁹ have shown previously that phenobarbitone pretreatment increased the cytochrome P-450 content of guinea pig microsomes and increased the activity of aniline hydroxylase. These authors also showed that, the decreased microsomal enzyme activities and decreased concentration of cytochrome P-450 in the ascorbic acid deficient guinea pig, could be returned to normal values after phenobarbitone pretreatment. The microsomal enzyme protein synthesising mechanism is therefore operable in ascorbic acid deficient guinea pigs. This has been confirmed in the present study.

Khanna et al¹²⁷ demonstrated an increase in MEOS after phenobarbitone treatment in rats, the increased activity was significant whether it was expressed per mg of microsomal protein or per g of liver. Lieber and DeCarli³⁸ and Mezey¹³¹ demonstrated an increase in MEOS after phenobarbitone, only when its activity was expressed per g of liver. Carter and Isselbacher³⁰ failed to show any change in MEOS after phenobarbitone, when its activity was expressed per mg of microsomal protein. These authors also found that NADPH oxidase activity was

unchanged by phenobarbitone treatment. The difference in these results could be due to variations in the duration, dose and route of administration of phenobarbitone between the studies. Although Khanna et al¹²⁷ did obtain an increase in MEOS activity after phenobarbitone treatment, there was no change in the rate of ethanol clearance from the blood of rats used in their study. The lack of effect of phenobarbitone on MEOS or NADPH oxidase activity in the guinea pigs of the present study, agrees with the above results obtained by Carter and Isselbacher³⁰ in rats.

In the present study aniline hydroxylase activity was increased by over 30 per cent, and cytochrome P-450 concentration was increased by over 60 per cent in the phenobarbitone treated guinea pigs, but MEOS activity and the rate of ethanol metabolism in vivo was unchanged. As MEOS activity is not induced by phenobarbitone, and is not dependent on the concentration of cytochrome P-450, it does not appear to have properties associated with 'true' microsomal enzymes.

No evidence has been found for an involvement of microsomal drug metabolizing enzymes in ethanol metabolism, as an increase in aniline hydroxylase activity and cytochrome P-450 concentration had no effect on ethanol metabolism in vivo.

CHAPTER 2.

HUMAN STUDIES

SECTION 1.

HEPATIC ALCOHOL DEHYDROGENASE ACTIVITY
AND LEUCOCYTE ASCORBIC ACID CONCENTRATION.

SECTION 1 (i)

RELATIONSHIP IN PATIENTS WITH NON-ALCOHOLIC
LIVER DISEASE.

Alcoholics suffer from many dietary deficiency states as their dietary intake is usually inferior in quality and quantity to that of non-alcoholics.¹³² Studies by O'Keane et al¹³³ have shown that ascorbic acid deficiency associated with an inadequate intake is common in alcoholics.

Leevy et al¹³⁴ have also demonstrated that hypovitaminosis is common in both alcoholic and non-alcoholic liver disease. This is due to dietary intake being inadequate to meet increased requirements imposed by tissue injury. Reduced levels of leucocyte ascorbic acid are frequently found in patients with liver disease.¹³⁵

Human alcohol dehydrogenase activity (ADH) is decreased in patients with histological evidence of liver disease.^{136,137,138} ADH activity is lowest in subjects with cirrhosis, especially where jaundice is also present.¹³⁷

Because of the link between nutritional status and liver disease, it was of interest to investigate whether the extent of liver disease, as determined by histology, could be related to the degree of hypovitaminosis. As ascorbic acid has been measured previously in guinea pigs, it was decided to measure this vitamin in humans, especially as it is reduced in alcoholics¹³³ and patients with liver disease.¹³⁵ Leucocyte ascorbic acid (LAA) concentration has been shown by Krebs et al¹³⁹ to be the best indirect method of assessing tissue ascorbic acid concentration.

As ADH activity is decreased in liver disease,^{136,137,138} the possibility of a relationship between ADH and LAA concentration was also investigated.

Methods.

Twelve patients with clinical or biochemical evidence of non-

alcoholic liver disease were studied. Liver tissue was obtained by Menghini needle biopsy with the informed consent of the patient. Half the biopsy was used for histological studies, and the other half for ADH determination. ADH activity and LAA concentration were measured as described previously. In addition liver function tests were performed, including serum aspartate and alanine transferase, bilirubin, alkaline phosphatase, and albumin and globulin levels.

Results.

In table 11, ADH activity, LAA content and the summary of the histological findings of the 12 patients are detailed. There was no obvious relationship between LAA concentration and histological findings, or between ADH activity and histology. Using the method of least squares, a highly significant linear correlation coefficient $r=0.88$ ($P<0.001$) was obtained when each patients ADH activity was plotted against its corresponding LAA concentration (fig. 12). No relationship was found between ADH activity and liver function tests.

Discussion

Although a highly significant correlation coefficient was obtained when ADH activity was plotted against LAA concentration, the nature of this relationship is unclear. ADH activity appears to be related to LAA concentration, but as this relationship was found in patients with liver disease, it is possible that it is simply a result of liver disease.

To establish the exact nature of this relationship, it would be of interest to see whether it also holds in subjects with no evidence of liver disease.

Table 11 ADH activity, LAA content and
liver histology in 12 patients
with non-alcoholic liver disease

ADH at pH 8.8 (U/g protein)	LAA ($\mu\text{g}/10^8\text{WBC}$)	Histology	Jaundice
20.7	34.6	Portal Cirrhosis	+
12.9	17.7	Infectious hepatitis (minimal changes)	+
12.8	26.7	Fatty infiltration (? toxic factor)	-
12.2	23.7	Mild non-specific inflammation	-
10.7	25.6	Biliary stasis - obstruction	+
8.9	19.0	Chronic aggressive hepatitis	+
8.4	7.2	Cirrhosis	+
5.8	7.0	Secondary carcinoma	-
5.7	11.3	Primary biliary cirrhosis	+
5.1	15.4	Chronic active hepatitis	-
4.9	10.5	Secondary carcinoma	+
2.2	6.3	Secondary Carcinoma	-

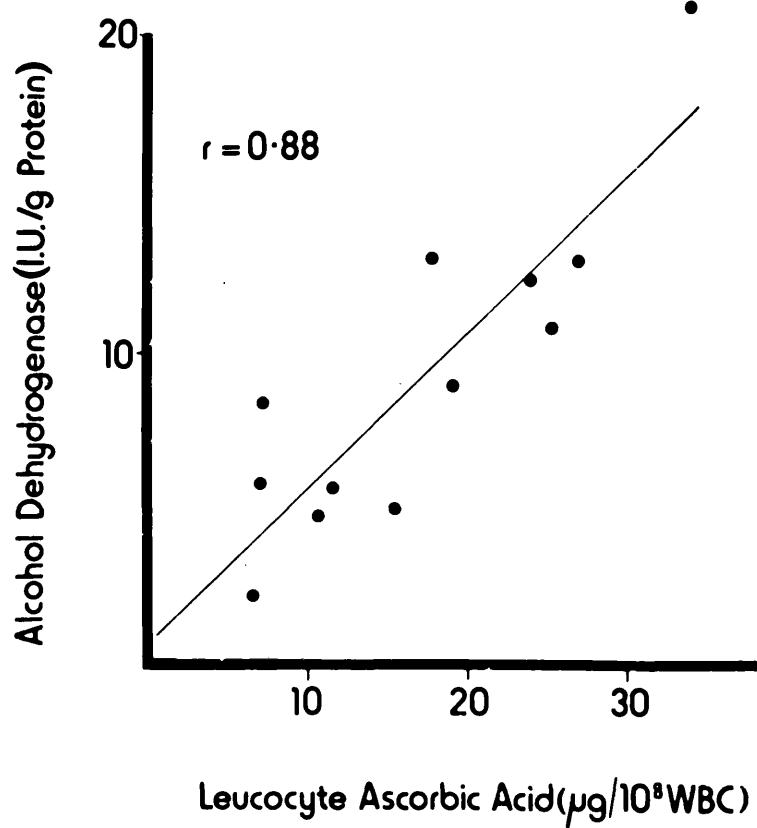


FIG. 12

Correlation between ADH activity and LAA content in

12 patients with non-alcoholic liver disease.

($P < 0.001$)

SECTION 1 (ii)

Ascorbic Acid and Ethanol Metabolism.

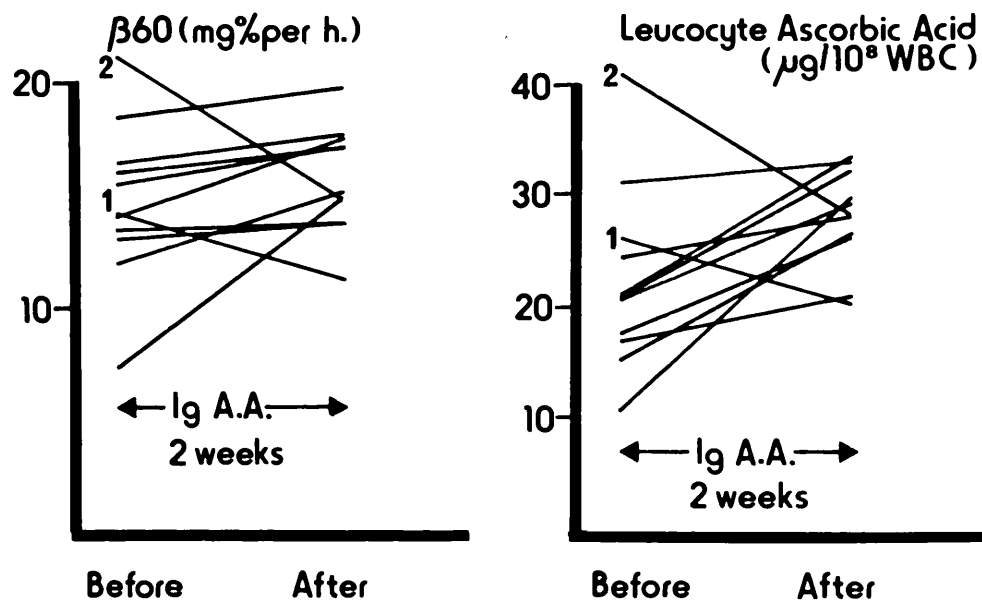
As it is unethical to subject healthy individuals to hepatic biopsy when there is no clinical evidence of liver disease, and hepatic histological results are not required for diagnosis, indirect methods must be used to establish whether ADH activity is related to ascorbic acid status in subjects with no evidence of liver disease. ADH is the initial and rate limiting enzyme in the major pathway of ethanol oxidation⁶, therefore any agent which affects its activity should alter the rate of ethanol clearance from the blood. The possibility of ascorbic acid status affecting the activity of ADH was therefore studied indirectly in healthy volunteers by measuring the rate of clearance of ethanol from the blood, before and after oral ascorbic acid.

Methods.

The rate of clearance of ethanol from the blood was measured in eleven healthy male volunteers, before and after treatment with an oral dose of 1g ascorbic acid daily for two weeks. The subjects fasted overnight prior to measurement of ethanol clearance rates. Between 9.30 a.m. and 10 a.m. they drank 150ml vodka diluted with lemonade, and blood samples were removed at hourly intervals for four hours. Blood ethanol and the rate of ethanol removed from the blood were determined as previously described. In humans the rate of ethanol cleared from the blood in mg ethanol/100ml blood/hour is termed the β_{60} ⁴⁶. LAA was also measured before and after ascorbic acid treatment.

Results.

Except in two subjects (nos 1 and 2), there was a significant increase in LAA concentration after ascorbic acid treatment ($P < 0.02$, fig 13). Similarly there was a significant rise in β_{60} after ascorbic acid



A.A. = ASCORBIC ACID

FIG. 13

$\beta 60$ and LAA content before and after oral
supplementation of Ig ascorbic acid for 2 weeks.

treatment, a significant increase was obtained ($P < 0.05$) after oral ascorbic acid. Using the method of least squares, a significant correlation coefficient was obtained when the $\beta 60$ of each subject was plotted against its corresponding LAA concentration ($r = 0.6$, $P < 0.005$; fig 14).

Discussion.

The significant increase in $\beta 60$ after ascorbic acid treatment indicates that increased LAA concentrations are associated with an increased rate of ethanol clearance from the blood. This could be due to increased activities of ADH, although the relationship found between ADH and LAA was only established in patients with liver disease, and may not necessarily hold in subjects with no evidence of liver disease.

From the results obtained in this study, it is impossible to exclude other factors that may be contributing to the increase in blood ethanol clearance rate. It is possible that other ethanol oxidizing enzymes may be increased by the ascorbic acid treatment, especially microsomal enzymes which have been previously shown to be affected by ascorbic acid status. Alternatively altered renal clearance of ethanol may be contributing to the increase in $\beta 60$.

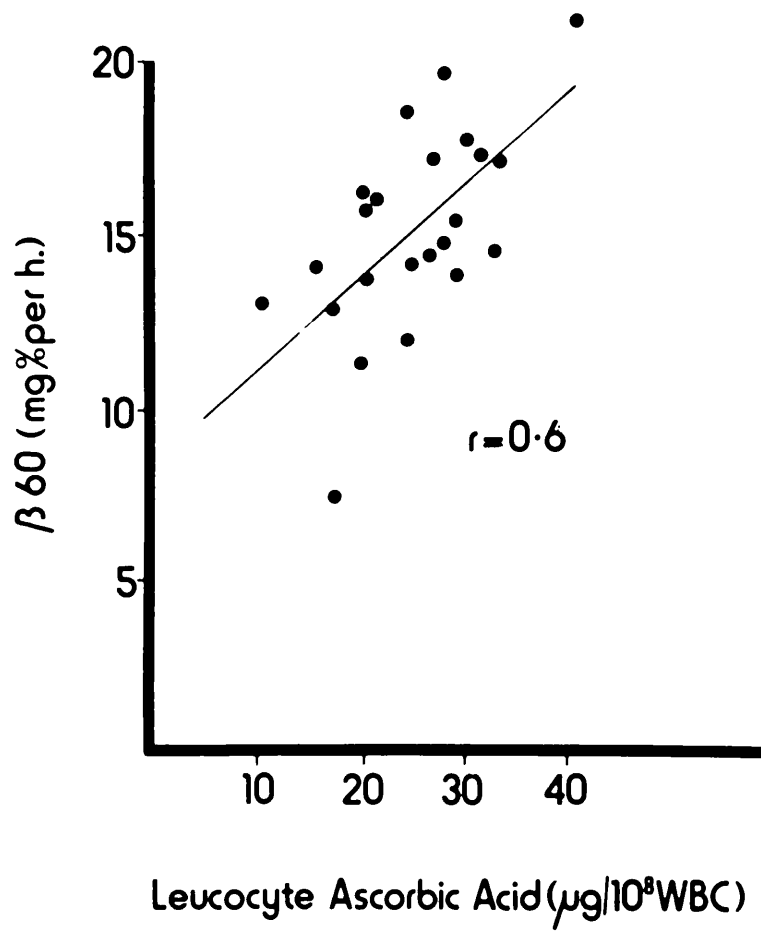


FIG. 14

Correlation between β 60 and LAA content. ($P < 0.005$)

SECTION 1 (iii)

Relationship in Patients With Alcoholic and Non-
Alcoholic Liver Disease and in Control Subjects.

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As liver biopsy samples could not be obtained from subjects with no evidence of liver disease, the nature of the above relationship between ADH and LAA is still unclear. The possibility of obtaining normal liver samples from patients with duodenal ulcer prompted the present study. Its purpose was to investigate a control group of patients, and establish whether the above relationship also held in patients with no evidence of liver disease. At the same time, a larger group of patients with liver disease were studied to see if this relationship was also evident in patients with alcoholic liver disease.

METHODS

Patients

Thirty-five patients with clinical or biochemical evidence of liver disease were studied, in whom a liver biopsy was indicated for diagnostic purposes. The biopsy was obtained using a Menghini needle and half the sample was fixed in formal saline for histological purposes and the other half placed on ice for alcohol dehydrogenase determination. The relevant history of alcohol intake was noted for each patient and they were divided into groups depending on this intake.

The classification of individuals with regard to their drinking history can only be done on a purely arbitrary basis. Individuals who regularly consumed over four pints of beer, or the equivalent in spirits, on more than four occasions a week were considered moderate drinkers. Those who indulged in almost daily consumption of large quantities of alcohol to the extent that their physical and/or mental well-being was impaired were placed in the alcoholic/heavy drinker group.

Control subjects.

Wedge liver biopsies were obtained during surgery for duodenal

ulcer in ten subjects with no clinical or biochemical evidence of liver disease; prior consent for the removal of the biopsies was obtained. No morphological changes were evident on histological examination of the liver biopsies; liver function tests were normal and there was no history of excessive alcohol consumption in these subjects.

Effects of in vitro ascorbic acid

Ascorbic acid solutions neutralised with NaOH and with final concentration in the range of 0.5mM to 10mM were added to the alcohol dehydrogenase assay system and alcohol dehydrogenase determined as above.

Biochemical tests of Liver Function

Liver function tests were performed on all the subjects studied. These included serum albumin, globulin and bilirubin levels, serum activities of alkaline phosphatase and alanine and aspartate transferases.

Statistical methods

The significance of results was tested using the Students t test and Mann-Whitney U test.

Results

Biochemical tests of hepatic function in the patients with liver disease, divided according to their drinking history, are given in tables 12, 13 and 14. The activity of hepatic alcohol dehydrogenase, concentration of leucocyte ascorbic acid and summary of hepatic histology for each of the patients with liver disease are given in tables 15, 16 and 17. In the non drinker group (table 15), there is one subject with alcohol dehydrogenase activity within the normal range of the present study (32-62 U/g protein) and 5 subjects whose leucocyte ascorbic acid concentration falls within the normal range ($18-50 \mu\text{g}/10^8 \text{ WBC}$). Low alcohol dehydrogenase activities are associated with low concentrations of leucocyte ascorbic acid in this group, but it is difficult to relate

Table 12 Biochemical tests of hepatic function
in 10 non drinkers with liver disease

Patient No.	AST ^a (iu/l)	ALT ^b (iu/l)	Bilirubin (mg/100ml)	Alkaline Phosphatase ^c	Albumin (g/100ml)	Globulin (g/100ml)
1	135	112	0.6	85	3.4	3.9
2	19	15	0.2	5	3.6	5.8
3	83	108	8.6	19	3.6	3.0
4	76	49	3.2	25	3.4	3.6
5	106	230	11.3	15	3.9	3.8
6	236	114	2.4	110	3.1	2.3
7	528	368	20.0	65	3.1	4.9
8	34	31	1.0	15	2.9	3.6
9	168	112	48	320	3.8	3.9
10	65	32	1.1	70	3.3	3.1
Normal Range	13-42	11-55	0.2-1.0	3-16	3.6-5.2	1.9-3.7
% Abnormal	80	60	70	70	60	50

a Aspartate transaminase

b Alanine transaminase

c King-Armstrong units

Table 13 Biochemical tests of hepatic function in 10
moderate drinkers with liver disease

Patient No.	AST ^a (iu/l)	ALT ^b (iu/l)	Bilirubin (mg/100ml)	Alkaline Phosphatase ^c	Albumin (g/100ml)	Globulin (g/100ml)
1	35	24	5.3	8	5.4	2.4
2	116	35	4.6	36	2.3	4.2
3	229	499	6.8	22	4.4	4.3
4	60	35	0.9	16	3.7	4.9
5	96	82	2.4	32	4.2	3.5
6	58	33	0.7	17	2.2	3.3
7	19	10	0.3	20	3.1	3.8
8	76	44	1.9	20	2.4	3.2
9	88	39	1.2	35	3.5	5.0
10	45	13	1.4	25	2.7	3.0
Normal Range	13-42	11-55	0.2-1.0	3-16	3.6-5.2	1.9-3.7
% Abnormal	80	30	70	80	70	50

a Aspartate transaminase

b Alanine transaminase

c King-Armstrong units

Table 14 Biochemical tests of hepatic function in 15
alcoholic/heavy drinkers with liver disease

Patient No.	AST ^a (iu/l)	ALT ^b (iu/l)	Bilirubin (mg/100ml)	Alkaline Phosphatase ^c	Albumin (g/100ml)	Globulin (g/100ml)
1	55	41	0.5	28	3	6.5
2	52	42	0.4	15	1.5	2.9
3	70	33	0.7	8	4.0	4.1
4	34	43	0.5	12	4.4	3.6
5	79	56	3.3	19	3.6	3.4
6	50	40	0.5	12	3.0	3.1
7	49	29	2.2	15	2.5	3.1
8	64	87	0.7	20	3.7	2.2
9	251	371	2.2	8	3.7	5.8
10	960	380	2.4	18	4.7	2.9
11	80	10	2.5	22	2.8	3.2
12	74	67	10.2	42	4.5	2.5
13	54	17	3.8	13		
14	68	25	6.7	41	2.0	4.2
15	90	100	30.4	17	2.5	4.5
Normal Range	13-42	11-55	0.2-1.0	3-16	3.6-5.2	1.9-3.7
% Abnormal	93	47	60	47	47	33

a Aspartate transaminase

b Alanine transaminase

c King-Armstrong units

Table 15 ADH activity, LAA levels and hepatic histology
in 10 non drinkers with liver disease

	Sex	ADH at pH 11.0 (U/g protein)	LAA ($\mu\text{g}/10^8\text{WBC}$)	Histology	Jaundice
1.	M	40.2	46.9	Primary biliary cirrhosis	-
2.	M	29.0	23.7	Mild non specific inflammation	-
3.	F	20.0	25.0	Viral hepatitis	+
4.	M	17.8	25.6	Biliary obstruction	+
5.	F	17.1	17.7	Infectious hepatitis	+
6.	M	16.3	7.2	Histiocytic infiltration	-
7.	F	16.1	19.0	Chronic aggressive hepatitis	+
8.	M	11.7	15.9	Fatty infiltration	-
9.	F	8.9	11.3	Primary biliary cirrhosis	+
10	M	1.1	6.3	Secondary carcinoma	-

Table 16 ADH activity, LAA levels, and hepatic histology in 10 moderate drinkers with liver disease.

	Sex	ADH at pH 11.0 (U/g protein)	LAA ₈ (μ g/ 10^8 WBC)	Histology	Jaundice
1.	M	37.2	41.5	Normal	+
2.	M	37.2	34.6	Cirrhosis	+
3.	M	29.0	15.7	Viral hepatitis	+
4.	M	25.3	29.0	Fatty infiltration	-
5.	M	22.4	12.3	Infectious hepatitis	+
6.	M	20.1	6.9	Fatty infiltration	-
7.	M	17.9	26.7	Fatty infiltration	-
8.	M	11.0	7.0	Cirrhosis	-
9.	F	10.2	15.4	Chronic active hepatitis	-
10.	F	5.5	9.1	Cirrhosis	+

Table 17 ADH activity, LAA levels, and hepatic histology
in 15 alcoholic/heavy drinkers with liver disease.

		ADH at pH 11.0	LAA		
	Sex	(U/g protein)	($\mu\text{g}/10^8$ WBC)	Histology	Jaundice
1.	M	42.6	19.3	Granulomatous condition	-
2.	M	34.2	26.5	Fatty infiltration	-
3.	F	32.6	24.9	Alcoholic hepatitis	-
4.	M	28.1	22.5	Portal cirrhosis	-
5.	M	27.3	23.0	Cirrhosis + alcoholic hepatitis	+
6.	M	25.0	32.0	Alcoholic hepatitis	-
7.	M	19.3	12.3	Fatty infiltration	+
8.	M	15.0	12.2	Fatty infiltration	-
9.	M	12.5	6.4	Cirrhosis	+
10.	M	12.2	24.0	Alcoholic hepatitis	-
11.	M	8.9	7.6	Acute + chronic alcoholic hepatitis	-
12.	M	5.9	4.0	Bile duct obstruction + Alcoholic hepatitis	+
13.	M	4.8	5.3	Cirrhosis + alcoholic hepatitis	+
14.	F	2.9	13.7	Cirrhosis + alcoholic hepatitis	+
15.	M	2.1	3.7	Advanced cirrhosis + acute alcoholic hepatitis.	+

these parameters to the extent of liver damage as assessed by histology.

In the moderate drinker group (table 16) two subjects have alcohol dehydrogenase activities within the normal range and four subjects have leucocyte ascorbic acid concentrations within the normal range. Again low alcohol dehydrogenase activities are associated with low leucocyte ascorbic acid concentrations, but there is no clear relationship between these two parameters and hepatic histological findings.

With regard to the alcoholic/heavy drinker group (table 17), three subjects have normal alcohol dehydrogenase activities and seven subjects have concentrations of leucocyte ascorbic acid within the normal range. Alcohol has contributed to hepatic pathological changes in this group, as there are nine subjects with histological changes consistent with alcoholic hepatitis and six of these individuals have the lowest activities of hepatic alcohol dehydrogenase in this group. Hepatic histological changes can be related to alcohol dehydrogenase activity, as the three lowest activities of alcohol dehydrogenase are found in subjects with cirrhosis and alcoholic hepatitis who were also jaundiced. No relationship between alcohol dehydrogenase activity and either serum aspartate transferase or serum alanine transferase was obtained.

In table 18, the alcohol dehydrogenase activity and leucocyte ascorbic acid concentration in the groups with liver disease are compared with values in the control subjects with no liver disease. There was no significant difference in alcohol dehydrogenase activity between the groups with liver disease, but alcohol dehydrogenase activity was significantly lower ($p < 0.001$) in these groups compared with the control group when the Students t test was applied. This was also significant using the Mann-Whitney U test ($p < 0.002$).

Table 18 Hepatic Alcohol Dehydrogenase Activity and Leucocyte Ascorbic Acid concentration in non drinkers, moderate drinkers and alcoholics/heavy drinkers with liver disease and in control subjects with no liver disease

	ADH pH 11.0 ($\frac{\mu}{\%}$ protein)	IAA (μ g ascorbic acid/ 10^8 WBC)
Non drinkers (n = 10)	17.8 \pm 10.7 *	19.9 \pm 11.8
Moderate drinkers (n = 10)	21.6 \pm 10.9 *	19.8 \pm 12.3
Alcoholics/Heavy drinkers (n = 15)	19.9 \pm 15.1 *	15.6 \pm 8.5 **
Control subjects (n = 10)	45.4 \pm 10.6	24.6 \pm 7.7

Each figure is the mean \pm S.D.

* differs by $p < 0.001$ from control

** differs by $p < 0.02$ from control

The leucocyte ascorbic acid concentration of the alcoholic/heavy drinker group was significantly lower ($p < 0.02$) than that of the control group, using the Students t test, but this was not significant when the Mann-Whitney U test was applied.

A scatter diagram obtained by plotting the alcohol dehydrogenase activities of the 35 patients with liver disease against their corresponding leucocyte ascorbic acid concentrations is illustrated in figure 15. A straight line correlation was found with $r = 0.77$ and a significance of $p < 0.001$. This was repeated with the liver disease patients divided according to their previous drinking histories and in each group a significant correlation coefficient was obtained (figure 16). Significant correlation coefficients of $r = 0.873$ ($p < 0.001$) for the non drinkers, $r = 0.739$ ($p < 0.02$) for the moderate drinkers and $r = 0.702$ ($p < 0.005$) for the alcoholic/heavy drinkers were obtained. An insignificant correlation coefficient of $r = 0.332$ was found for the control subjects with no liver disease.

The in vitro addition of ascorbic acid in the range of 0.5mM to 10mM final concentration had no effect on alcohol dehydrogenase activity.

Discussion

The low hepatic alcohol dehydrogenase activities found in the groups of patients with liver disease is in agreement with the results of Figueroa and Klotz¹³⁷ who demonstrated low alcohol dehydrogenase activities in patients with cirrhosis and jaundice. In the present study, when alcohol dehydrogenase activity, leucocyte ascorbic acid concentration and extent of liver damage (as assessed by hepatic histology) are considered in each patient with liver disease, it is apparent that patients with changes in liver histology associated with

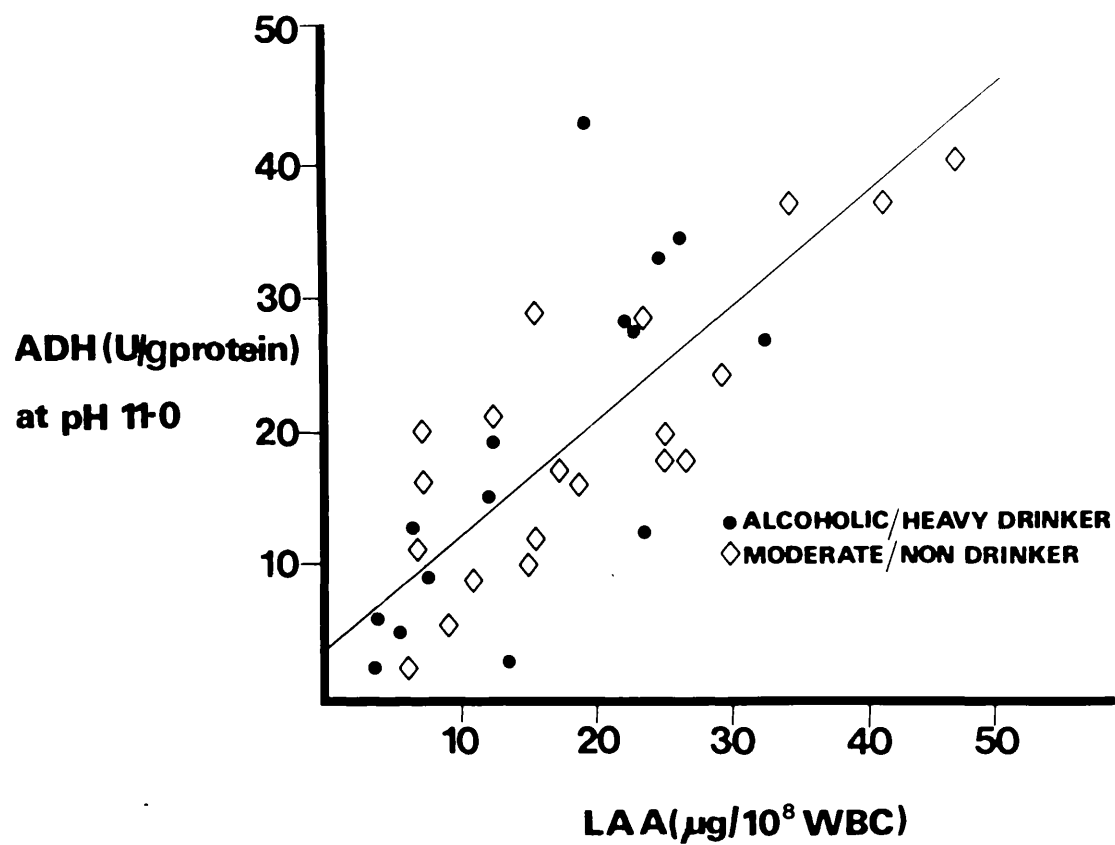


FIG. 15

Correlation between ADH activity and LAA content in

35 patients with liver disease. ($r=0.77$, $P<0.001$)

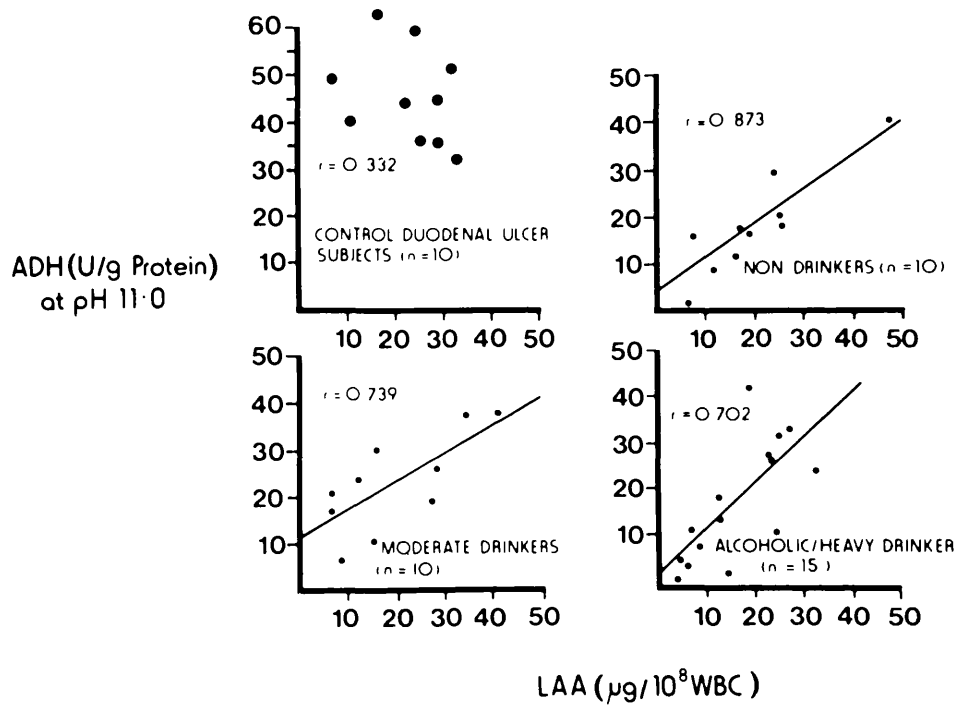


FIG. 16

Correlation between ADH activity and LAA content in 10 control subjects and in 35 patients with liver disease, divided according to their previous alcohol intake.

cirrhosis and alcoholic hepatitis who are also jaundiced, generally have low alcohol dehydrogenase activities and concomitant low concentrations of leucocyte ascorbic acid. It is possible that jaundice itself may interfere with ADH, as bilirubin has been shown to inhibit ADH in vitro¹⁴⁰.

As alcohol dehydrogenase activity in the moderate and non drinker groups is similar to that in the alcoholic/heavy drinker group, alcohol consumption per se does not seem to alter alcohol dehydrogenase activity.

In the present study the concentration of leucocyte ascorbic acid in the alcoholic/heavy drinker group is significantly decreased compared to that of the control group when the Students t test is applied, but there is no significant difference in leucocyte ascorbic acid concentration between the moderate and non drinker groups and the control group. The Mann-Whitney U test failed to demonstrate a significant difference between the leucocyte ascorbic acid concentrations of the groups with liver disease and the control group. This suggests that the significant difference found between the leucocyte ascorbic acid concentration of the alcoholic/heavy drinker group and the control group, when the Students t test was applied, is of little real significance. As the control group consists of subjects with duodenal ulcers, who tend to have lower than normal leucocyte ascorbic acid concentrations¹⁴¹, it is possible that this may contribute to the insignificant difference in leucocyte ascorbic acid concentration between the groups studied.

Although ascorbic acid deficiency is associated with decreased activities of microsomal drug hydroxylating enzymes and electron transport components in guinea pigs¹⁰⁹, the nature of the relationship between hepatic alcohol dehydrogenase and leucocyte ascorbic acid is unclear. In the patients with liver disease (figure 15), there is a significant correlation between alcohol dehydrogenase activity and the

concentration of leucocyte ascorbic acid, but this is not found for the control subjects. The range of the activity of alcohol dehydrogenase in the control subjects is approximately 32 - 62 U/g protein, whereas in the liver disease patients it is approximately 1 - 45 U/g protein (figure 16). The insignificant correlation found between hepatic alcohol dehydrogenase activity and the concentration of leucocyte ascorbic acid in the control group, may be explained by this difference in the range of activities of alcohol dehydrogenase between the control and liver disease groups, although the presence of liver disease may be the determining factor.

In vitro addition of ascorbic acid in the concentrations described has no effect on alcohol dehydrogenase activity; consequently ascorbic acid does not seem to be involved in the alcohol dehydrogenase reaction. This has been confirmed previously in vivo, as there is no correlation between alcohol dehydrogenase activity and ascorbic acid status in the ascorbic acid deficient guinea-pig. The presence of liver disease is therefore an important factor in this correlation found in the human.

The relationship between alcohol dehydrogenase activity and the concentration of leucocyte ascorbic acid also holds when the subjects with liver disease are divided according to their previous alcohol intake (figure 16). Alcohol intake does not seem to be responsible for this relationship as a significant correlation is also found in the non drinker group.

It is concluded that the correlation between alcohol dehydrogenase activity and leucocyte ascorbic acid concentration found in the present work is probably a consequence of liver disease, as opposed to any specific effect of ascorbic acid status or alcohol consumption on alcohol dehydrogenase activity.

SECTION 2

Incidence of Atypical ADH in a
Scottish Population.

Human ADH exists in multimolecular forms, at least seven isoenzymes can be distinguished by electrophoretic and chromatographic methods¹⁵. Schenker et al¹⁶ have isolated, purified and determined the subunit composition of six of these isoenzymes. These authors have suggested that the isoenzymes are formed by combinations of three subunits designated A, B and B¹ to give six dimers AA, BB, AB, BB¹, B¹B¹ and AB¹. Berger et al¹⁷ have shown that subunit B can exist in two forms termed B₁ and B₂. These subunits differ in one amino acid residue, which is located in a region of the subunit which corresponds to the coenzyme binding site of horse ADH. B₂ is considered the subunit which is responsible for the atypical catalytic properties discussed below.

Von Wartburg et al¹⁸ have shown that ADH exhibits a polymorphism, which is revealed by a bimodal distribution of the enzyme in a population. A screening test has been devised to differentiate between the normal and atypical enzyme¹⁹. The normal enzyme has a pH optimum of 10.8 while the atypical enzyme has a pH optimum of 8.5.

The screening test involves measurement of ADH activity at pH 8.8 and pH 11.0. The quotient formed by dividing the activity at pH 11.0 by that at pH 8.8, known as Q pH, is used to differentiate between the normal and atypical enzyme. Q pH is greater than one for the normal enzyme, and less than one for the atypical enzyme.

The incidence of atypical ADH in a Scottish population was studied using the above screening method.

Methods.

ADH activity was measured at pH 8.8 and pH 11.0, as described previously, in liver biopsy samples from the 35 patients with liver disease, and the 10 subjects with duodenal ulcer, who were studied in

the previous section. The frequency distribution of Q pH was plotted for the 45 samples.

Results.

The results of the screening test on the 45 liver biopsy specimens are shown in figure 17. Two subjects with atypical ADH were found, as there are two biopsies with $Q\ pH < 1$. Of the two subjects with the atypical form of ADH, one was from the control group with no evidence of liver disease, and the other was from the group with liver disease. Q pH follows a bimodal distribution.

Discussion.

The frequency of atypical ADH in the Scottish population studied was 2 out of 45 subjects (fig. 17), which is approximately a 4% incidence. This agrees with a study of a London population, which gave a frequency of 2 out of 50 subjects with atypical ADH¹⁹. These same workers showed that atypical ADH was present in 20% of a Swiss population (12 out of 59 subjects)¹⁹.

Edwards and Evans⁴⁹ revealed a slightly higher incidence of the atypical enzyme in a Liverpool population (approximately 8%), but as this only represented 2 out of 23 subjects, the small population studied may have contributed to this apparent higher incidence.

The bimodal distribution of the atypical enzyme found in the London and Swiss populations by Von Wartburg and Schurch¹⁹, and in the Scottish population in the present study differs from that obtained by Ugarte et al¹⁴² in 80 Chilean alcoholics. These authors showed a trimodal distribution of Q pH and also suggested that atypical ADH is more common in alcoholics with steatosis or cirrhosis, compared with alcoholics with normal histology, unfortunately the statistical significance of these results is questionable. In the present study, no increased

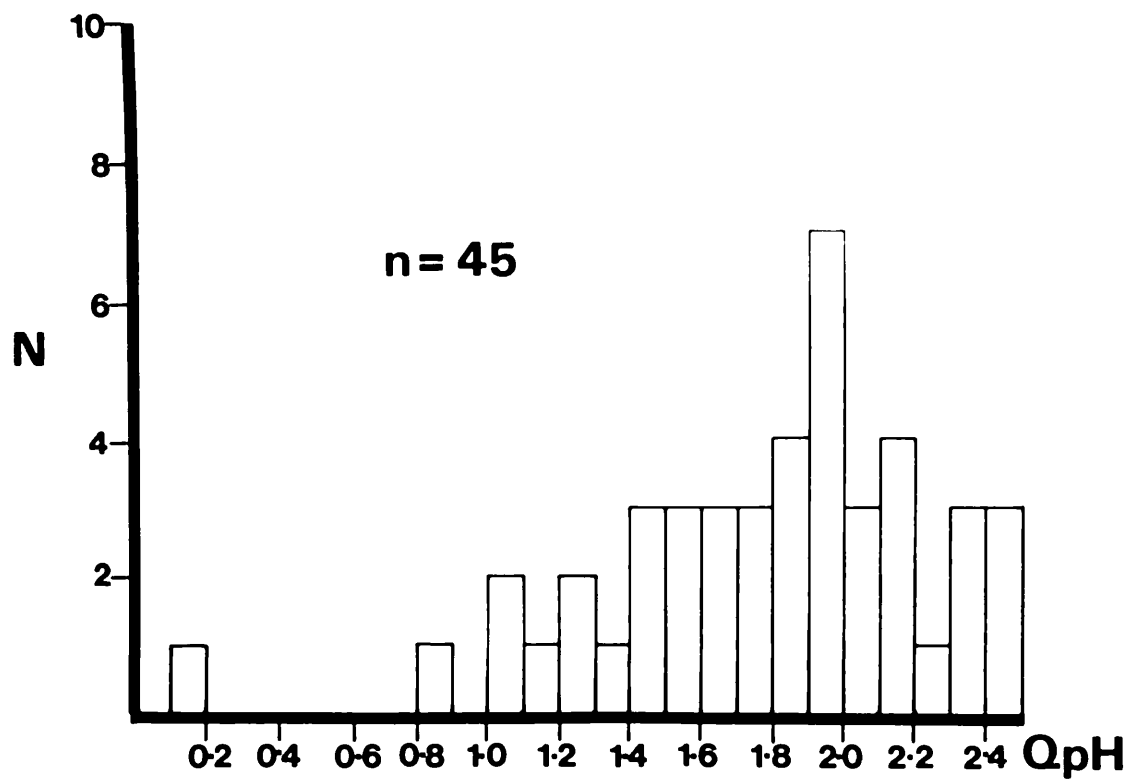


FIG. 17

Frequency distribution histogram of atypical ADH

in 45 liver biopsies from a Scottish population.

QpH < 1 for atypical ADH.

incidence of the atypical enzyme was evident in the patients with liver disease, although the population studied is probably too small to observe any possible increased incidence.

It is of interest that Ugarte et al¹⁴² found a trimodal distribution of normal and atypical ADH in their study, as Smith et al¹⁴³ have proposed a genetic model in which three groups of individuals would be expected from considerations of subunit composition. Their model suggests that two alleles occur at the gene locus coding for subunit B, and these alleles are responsible for the synthesis of either the normal (B_1) or atypical (B_2) subunit. Three groups of individuals are expected with respect to their B subunit composition, homozygotes with subunit B_1 only, homozygotes with subunit B_2 only and heterozygotes with subunits B_1 and B_2 .

Berger et al¹⁷ have confirmed this proposed genetic model by showing that isoenzyme BB, isolated from both normal and atypical phenotypes, contains subunits B_1 and B_2 in the atypical isoenzyme, and only subunit B_1 in the normal isoenzyme. B_2 is therefore responsible for the atypical characteristics. Isoenzymes BB from human liver can therefore contain three isoenzymes with the composition B_1B_1 , B_1B_2 or B_2B_2 . This agrees with the genetic model of Smith et al¹⁴³. In view of the low estimated frequency of the allelic gene b_2 , an extremely low frequency of homozygous individuals with genotype b_2b_2 and isoenzyme composition B_2B_2 would be expected. Large numbers of liver samples would therefore need to be screened before this trimodal distribution can be detected.

SECTION 3.

ETHANOL OXIDIZING ENZYMES
IN PATIENTS WITH LIVER DISEASE

The possible involvement of MEOS in ethanol metabolism has been previously studied in man by Mezey and Tobon¹⁴⁴, who measured the rate of ethanol clearance from the blood and the activities of ADH and the NADPH dependent ethanol oxidizing system, in recently drinking alcoholic patients without liver disease and in non-drinking controls. The rate of ethanol clearance and the activity of the NADPH dependent ethanol oxidizing system were increased in the alcoholic patients; but although the rate of ethanol clearance returned to normal after 7 days abstinence from ethanol, the activity of the NADPH dependent ethanol oxidizing system only decreased significantly after 21 days abstinence from ethanol.

Non parallel changes in ethanol clearance rates and the activity of the NADPH dependent ethanol oxidizing system have also been shown in phenobarbitone treated alcoholic patients¹⁴⁵ who had no evidence of liver disease. ADH activity and NADPH dependent ethanol oxidizing system activity, as well as ethanol clearance rates, were measured in alcoholics before and after phenobarbitone administration. Ethanol clearance rates were increased, but no change in the activities of ADH or NADPH dependent ethanol oxidation was apparent.

Previous investigators have shown a decrease in ADH activity in patients with liver disease¹³⁷, but no measurement of the other enzymes involved in ethanol metabolism was undertaken. In the present study, ADH, catalase and the NADPH dependent ethanol oxidizing system were measured in hepatic biopsies from patients with liver disease, and the activities compared with those in control subjects with no liver disease.

Methods

Patients

Nine patients with clinical or biochemical evidence of liver disease were studied; in each case a liver biopsy was indicated for diagnostic purposes. The history of alcohol consumption of each patient was noted. No patient had consumed alcohol for at least one week prior to biopsy. Patients receiving drugs known to induce hepatic microsomal enzymes were excluded.

Control subjects

Liver biopsies were obtained during surgery for duodenal ulcer with the informed consent of 7 subjects with no clinical or biochemical evidence of liver disease. Histology showed no morphological changes in the liver, and no history of excessive alcohol consumption was found in this group of patients.

Enzyme determinations

ADH, catalase and NADPH dependent ethanol oxidation were measured in each biopsy, using previously described methods.

Results

The history of alcohol consumption, biochemical tests of liver function and hepatic histology of the 9 patients with liver disease are summarised in Table 19. Histology indicated that alcohol played a major role in the pathological changes in the livers of patients with an alcoholic or heavy drinking history.

The activities of the ethanol oxidizing enzymes measured in the 7 control subjects and 9 patients with liver disease are given in Tables 20 and 21 respectively. Control subject number 4 is a carrier of the atypical form of ADH (Table 20). The activities of the ethanol oxidizing enzymes in the two groups are compared in Table 22, with

Table 19 Hepatic Histology, Drinking History, and Biochemical Tests of Hepatic Function in 9 Patients with Liver Disease.

Patient	Sex	Drinking History*	AST ^a (I.U./l)	ALT ^b (I.U./l)	Bilirubin (mg/100ml)	Alkaline Phosphatase (K.A. ^c units)	Albumin (g/100ml)	Globulin (g/100ml)	Histology
A.P.	M	H	90	100	30.4	17	2.5	4.5	Alcoholic hepatitis + advanced cirrhosis
J.M.	M	A	79	56	3.3	19	3.6	3.4	Alcoholic hepatitis + early cirrhosis + obstructive jaundice
J.McD	M	A	52	42	0.4	15	1.5	2.9	Mild fatty infiltration + increased iron
M.H.	F	N.D.	83	108	8.6	19	3.6	3.0	Acute viral hepatitis
A.McA	M	N.D.	34	31	1.0	15	2.9	3.6	Bile duct obstruction
D.G.	F	N.D.	135	112	0.6	85	3.4	3.9	Chronic active hepatitis
M.J.	M	H	34	43	0.5	12	4.4	3.6	early portal cirrhosis
D.C.	M	A	192	54	7.4	24	2.2	3.4	cirrhosis + alcoholic hepatitis
W.A.	M	H	60	100	3.0	12.5	3.4	2.3	Early cirrhosis + mild alcoholic hepatitis
Normal Range			13-42	11-55	0.2-1.0	3-16	3.6-5.2	1.9-3.7	

^a Aspartate Transaminase

^b Alanine Transaminase

^c King-Armstrong Units

* H = heavy drinker

A = alcoholic

N.D. = non drinker

Table 20 Ethanol Oxidizing Enzymes in 7 Control Subjects

	ADH(U/g protein)		ADH(U/g liver)		NADPH dependent (n moles/ mg protein/min)		NADPH dependent (n moles/ g liver/min)		Catalase (μ moles/ mg protein/min)		Catalase (m moles/ g liver/min)	
	Sex	pH 8.8	pH 11.0	pH 11.0								
1.	M	24.6	49.2	7.3	3.8	566.5	36.2	5.4				
2.	F	18.5	32.1	4.9	4.2	644.6	15.9	2.4				
3.	M	17.2	44.3	5.6	5.1	650.1	27.2	3.5				
4.	M*	48.4	44.0	7.9	1.3	217.6	46.5	6.2				
5.	M	19.9	35.7	5.2	9.2	1344.9	34.2	5.0				
6.	M	24.5	51.4	5.2	4.6	459.3	56.0	6.7				
7.	M	22.5	35.2	6.0	3.7	628.9	47.4	8.1				

* atypical ADH

Table 21 Ethanol Oxidizing Enzymes in 9 patients
with liver disease

Sex	ADH(U/g protein)		ADH(U/g liver)		NADPH dependent (nmoles/mg protein/min)		NADPH dependent (n moles/g liver/min)		Catalase (μ moles/ mg protein/min)		Catalase (m moles/ g liver/min)	
	pH 8.8	pH 11.0	pH 11.0	pH 11.0								
1. M	1.5	2.1	0.3	12.2	1700.3	8.4	0.9					
2. M	21.1	27.3	1.7	5.8	2070.7	13.9	1.6					
3. M	18.6	34.2	7.6	5.3	1354.4	33.5	4.5					
4. F	10.2	19.5	3.5	8.9	1164.1	26.0	4.7					
5. M	6.1	11.7	1.2	13.2	1367.3	21.6	2.2					
6. F	19.2	40.2	5.3	13.8	1806.5	25.7	3.4					
7. M	14.1	28.1	6.9	4.0	983.8	43.0	4.1					
8. M	3.5	5.5	0.6	6.1	636.7	16.4	1.9					
9. M	4.5	5.5	0.7	8.6	1045.8	13.3	1.6					

enzyme activities expressed in terms of protein concentration. Alcohol dehydrogenase and catalase activities were significantly lower, and the activity of the NADPH dependent ethanol oxidizing system was significantly higher in the patients with liver disease, when the Student's t test was applied. The activity of the NADPH dependent ethanol oxidizing system was also significantly higher in the liver disease group than that in the control subjects, when the Mann-Whitney U test was used ($P < 0.02$).

The mean activity of the NADPH dependent ethanol oxidizing system found in the control subjects of the present work (4.6 ± 2.4 nmoles/mg protein/min) is similar to that found by Mezey and Tobon¹⁴⁴ in their control subjects (4.2 ± 1.0 nmoles/mg protein/min).

The activities of the ethanol oxidizing enzymes in the control and liver disease groups are compared in Table 23, with enzyme activities expressed in terms of liver weight. ADH and catalase activities were significantly lower, and the activity of the NADPH dependent ethanol oxidizing system was significantly higher in the patients with liver disease.

Discussion

Although liver biopsy samples are of relatively small size (20-40 mg), histological findings are generally representative of changes in the whole liver. Soloway et al¹⁵⁷ have demonstrated that the sampling error in multiple simultaneous biopsies was very small in cases of hepatitis, although this error was larger in cases of cirrhosis.

In the analysis of enzyme activities in liver biopsies the choice of reference for expressing enzyme activity is important. The activity can be related to wet weight, protein or RNA or DNA content, or to the number of cells in the biopsy specimen. In the present study enzyme

activities were expressed in terms of both wet weight and protein content of the liver biopsy specimen. The method of Lowry et al¹⁰⁰ used for the determination of the protein content of the biopsy specimens measures the number of tyrosine residues in a protein. Fibrotic tissue contains very little tyrosine¹⁵⁸ and will therefore not interfere with the calculation of enzyme activity, when it is expressed in terms of protein content. It is however useful to use more than one reference for the calculation of enzyme activities as this will minimise error in the interpretation of results from liver biopsies of patients with different types of liver disease.

A limitation of cytochemical enzyme determination in liver biopsy specimens is that no information is obtained about the localisation of enzyme activity in the tissue. Some cells may have a high enzyme content and thus give a false picture of the overall activity in the liver. Histochemical methods are useful in that they can localise the activity of enzymes but unfortunately yield poor quantitation.

Although previous investigators have shown non parallel changes in the rates of ethanol clearance from the blood and the activity of the NADPH dependent ethanol oxidizing system,^{144, 145} they have mainly been concerned with the mechanism of increased ethanol clearance rates. A possible mechanism for the maintenance of normal rates of ethanol clearance in patients with low ADH activities, is considered below.

The decrease in ADH activity shown in the patients with liver disease is in agreement with previous work of Figueroa and Klotz¹³⁷. ADH

Table 22 ETHANOL OXIDIZING ENZYMES IN CONTROL SUBJECTS
AND PATIENTS WITH LIVER DISEASE *

	Alcohol Dehydrogenase pH 8.8 (U/g protein)	Alcohol Dehydrogenase pH 11.0 (U/g protein)	Catalase (μ moles/mg protein/min)	NADPH Dependent Ethanol Oxidising System (n moles/mg protein/min)
Patients with liver disease (N = 9)	11.0 \pm 7.5	19.3 \pm 13.9	22.4 \pm 11.0	8.7 \pm 3.7
Control subjects (N = 7)	25.1 \pm 10.7	41.7 \pm 7.5	37.6 \pm 13.6	4.6 \pm 2.4
Significance P <	0.01	0.005	0.05	0.025

* Results expressed as mean \pm S.D.

Table 23 Ethanol Oxidizing Enzymes in Control Subjects
and Patients with Liver Disease

	Alcohol Dehydrogenase pH 11.0 (U/g liver)	Catalase (m moles/g liver/min)	NADPH dependent ethanol oxidizing system (n moles/g liver/min)
Patients with liver disease (n = 9)	3.1 ± 2.9	2.8 ± 1.4	1347.7 ± 450.1
Control subjects (n = 7)	6.0 ± 1.2	5.3 ± 1.9	644.6 ± 344.8
Significance p <	0.025	0.01	0.005

activity is decreased by approximately 50% in the nine patients with liver disease. Although it is difficult to relate in vitro enzyme activities with in vivo activities, this decrease may affect ethanol clearance rates as Goldberg and Rydberg²² have shown that inhibition of rat ADH with pyrazole reduces ethanol metabolism in vivo. Ethanol clearance rates were not measured in the present study, but other investigators have shown that ethanol clearance rates are unchanged in patients with liver disease.^{146, 147}

There are two possible mechanisms for the maintenance of ethanol clearance rates in patients with liver disease. The first possibility is, that even with a 50% reduction in ADH activity measured in vitro, there is sufficient activity in vivo to maintain normal rates of ethanol metabolism. The second possibility is that other enzymes involved in ethanol oxidation may play a quantitatively more important role in patients with liver disease. The results obtained in the present study add weight to this second possibility.

In the proposed system of Carter and Isselbacher,⁴² NADPH dependent ethanol oxidation takes place via NADPH oxidase activity which produces H_2O_2 , this in turn is used in the peroxidative conversion of ethanol to acetaldehyde, catalyzed by catalase. The decrease in catalase activity shown in the liver disease group is probably not great enough to affect its utilisation of H_2O_2 , as in the above proposed system NADPH dependent ethanol oxidation is regulated by the activity of NADPH oxidase and its production of H_2O_2 ³¹. The increase in NADPH dependent ethanol oxidation in the liver disease patients may reflect an increase in the activity of NADPH oxidase.

There are several possible explanations for the increased activity

of the NADPH dependent ethanol oxidizing system found in patients with liver disease. In alcoholic hepatitis liver protein is decreased,¹⁴⁸ and this could explain the increased activity of the NADPH dependent ethanol oxidizing system, when its activity is expressed in terms of protein concentration. Although, when its activity is expressed in terms of liver weight, it is still increased and differences in liver protein concentration are therefore unlikely to contribute to this increased activity.

Recent alcohol intake has also been shown to increase the activity of the NADPH dependent ethanol oxidizing system,¹⁴⁴ but this again is an unlikely explanation for the increased activity found in the present study. There are three non drinkers in the liver disease group (subjects 4, 5 and 6, Table 21) who also have high activities of the NADPH dependent ethanol oxidizing system.

The increase in the NADPH dependent ethanol oxidizing system may therefore be an adaptive increase because of low ADH activities in patients with liver disease, or may simply reflect increased cellular disruption.

Schoene et al¹⁴⁹ have measured cytochrome P-450 content and the activity of aminopyrine demethylase in human liver biopsies. They demonstrated that cytochrome P-450 content was decreased by 50% in severe hepatitis and cirrhosis. Similarly the rate of demethylation of aminopyrine was significantly reduced in severe liver disease. As cytochrome P-450 dependent systems are reduced in liver disease, it appears that the NADPH dependent ethanol oxidizing system is not cytochrome P-450 dependent and cannot be considered a 'true' microsomal enzyme.

General Discussion

Assuming that an alcoholic consumes one bottle of spirits per day over a period of several weeks during a drinking bout, the resulting ethanol intake is 4.3 g/Kg body wt/day for a 70 Kg man. This is calculated from the ethanol content of the spirit (40 g/100ml), and assumes that the bottle contains 750 ml spirit. As the LD_{50} for a single oral dose of ethanol is 4 g/Kg in the guinea pig⁴⁵, ethanol pretreatment was achieved by administering an oral dose of 2.5 g ethanol/Kg body wt/day for 14 days. Although the guinea pigs did not ingest ethanol throughout the day, as would the human alcoholic, the ethanol pretreatment they were given was as high and as near as possible to the intake of an alcoholic.

The above ethanol pretreatment resulted in a significant reduction in the extent of the redox state shift after ethanol administration, compared with that in unpretreated control guinea pigs (tables 3,4). This was not due to differences in blood ethanol concentrations between the ethanol pretreated and unpretreated control animals, as the theoretical zero time concentration of ethanol in the blood (C_0) was the same in both groups of animals (table 7). Although the redox state is important in the regulation of ethanol elimination in vivo¹¹⁵, the approximately ten per cent decrease in the extent of the redox state shift after ethanol administration to ethanol pretreated guinea pigs, was too small to influence their rate of ethanol elimination in vivo (table 7).

The decrease in the extent of the redox state shift after ethanol administration, demonstrated in ethanol pretreated guinea pigs, may represent metabolic adaptation to ethanol at the mitochondrial level. Previous work by Rawat and Kuriyama⁵³ showed that ethanol pretreated mice have an increased mitochondrial permeability to NADH. Videla et al³¹ have also demonstrated that mitochondria isolated from ethanol

pretreated rats have a faster rate of oxygen consumption, which suggests that they can reoxidise NADH at a faster rate. Although it is uncertain which of these mechanisms is operating in the ethanol pretreated guinea pig, both will result in an increased rate of mitochondrial utilization of NADH. Reducing equivalents produced from ethanol oxidation will therefore be metabolized at a faster rate, resulting in the observed attenuation of the redox state shift in the present study.

As metabolic adaptation was achieved in guinea pigs after a relatively short period of exposure to ethanol, it is very likely that this can occur in man after exposure to comparable quantities of ethanol.

The relationship between ADH activity and LAA content found in patients with liver disease (figures 12, 15) was not found in subjects with normal liver histology (figure 16). As the addition of ascorbic acid in vitro to the human ADH reaction system had no effect on its activity, it is concluded that this relationship is a consequence of liver disease as opposed to any specific effect of ascorbic acid status on ADH activity. This is further confirmed by the observation that ADH activity of normal and ascorbic acid deficient guinea pigs is not related to their ascorbic acid status (table 5).

When ADH activity, LAA content and hepatic histology are compared in patients with liver disease, it is apparent that patients who are jaundiced and have changes in liver histology compatible with cirrhosis and alcoholic hepatitis, have very low ADH activities and LAA contents (table 17). This may be useful in the diagnosis of chronic liver disease, when a liver biopsy cannot be carried out. If a patient has jaundice and suspected liver disease, demonstrated by abnormal biochemical liver function tests, a very low LAA is a good indication of chronic liver disease.

As ADH does not depend on ascorbic acid for activity, it is difficult to explain the increased rate of ethanol clearance from the blood ($\beta 60$) in man, after ascorbic acid supplementation (figure 13). As this increased rate is small and only represents 1-2 mg ethanol/100 ml blood/hr, it may simply reflect an altered renal clearance of ethanol. Alternatively it may be due to the enhanced activity of microsomal enzymes capable of oxidizing ethanol, as microsomal enzyme activities have been shown to be dependent on ascorbic acid status¹⁰⁹, although ethanol clearance rates were not enhanced in ascorbic acid supplemented guinea pigs compared with ascorbic acid deficient animals (table 7). The difference in the effect of ascorbic acid on ethanol metabolism in man, and the guinea pig, may be due to variations in the extent of tissue saturation with ascorbic acid after oral supplementation. Tissue saturation with ascorbic acid is attained at 260 μg ascorbic acid/g wet wt liver in guinea pigs¹⁰⁷ and 50 $\mu\text{g}/10^8$ WBC in man⁹⁹; from table 5 and figure 13 it is obvious that tissue saturation had not been reached in either the human volunteers or guinea pigs.

Although it is difficult to relate the in vitro activity of an enzyme to its possible in vivo activity, the increase in $t_{1/2}$ of diphenylhydantoin in the ascorbic acid deficient guinea pig (table 1) is probably due to a decrease in activity of microsomal drug metabolizing enzymes. This is confirmed in table 6, where aniline hydroxylase activity and cytochrome P-450 content are shown to be decreased in ascorbic acid deficient guinea pigs. Wade et al¹²³ showed that the decrease in activity of microsomal enzymes, isolated from ascorbic acid deficient guinea pigs, was not due to qualitative changes in the enzymes, as there was no change in K_m for aniline or hexobarbitone.

The 36% increase in $t_{1/2}$ of DPH in the ascorbic acid deficient guinea pig (table 1), compares with a 22% decrease in the activity of aniline hydroxylase and a 24% decrease in cytochrome P-450 content (table 6).

Unlike DPH, which is metabolized entirely by the microsomal drug metabolizing system, Lieber and De Carli¹⁵⁰ consider that 20% of ethanol metabolism in vivo is carried out by MEOS. As the increase in DPH $t_{1/2}$ is due to decreases in microsomal enzyme activities, a similar 36% decrease in the rate of ethanol elimination by microsomal pathways will only give rise to a 7.2% decrease in the overall rate of ethanol metabolism. This represents a decrease of approximately 2.1 mg/100 ml/hr in the rate of ethanol elimination from the blood in the normal guinea pig, whose rate of ethanol elimination is 29.3 ± 2.7 mg/100 ml/hr (table 7). Unfortunately this decrease is within the S.D. of the measurement of the rate of ethanol elimination in vivo and would probably not be detected. A further problem is that MEOS activity in vitro was unchanged in the ascorbic acid deficient animal. From these theoretical considerations it is therefore difficult to arrive at any firm conclusions regarding the extent of MEOS activity in vivo.

The in vivo significance of MEOS has been studied using inhibitors of the microsomal drug metabolizing system, in an attempt to establish the extent of microsomal involvement in ethanol metabolism. Khanna and Kalant¹²⁰ using the microsomal enzyme inhibitor SKF 525-A showed that it had no effect on the rate of ethanol clearance from the blood of rats and the whole body of mice. These authors concluded that ethanol was therefore not metabolized to any significant extent by microsomal enzymes. The above findings are questionable, as some drugs (especially those with type II binding characteristics) are metabolized by enzymes which are relatively insensitive to this inhibitor¹⁵¹. Rubin et al³⁷ have shown that ethanol produces a modified type II binding spectrum. Lieber and De Carli¹⁵² have also shown that MEOS activity is not affected by SKF 525-A in vitro, and conclude that work using this inhibitor to establish the extent of microsomal involvement in ethanol metabolism in vivo is invalid.

In the present study the use of non specific inhibitors has been avoided, with the use of the ascorbic acid deficient guinea pig as an animal model for studies on the effect of decreased microsomal enzyme activities on ethanol metabolism. Although in vivo studies were inconclusive, measurement of cytochrome P-450 content and aniline hydroxylase activity in vitro showed a significant decrease in the ascorbic acid deficient guinea pig, whereas MEOS activity was unchanged (table 6). As aniline is a type II substrate¹⁵³ and aniline hydroxylase is dependent on cytochrome P-450 for its activity, ethanol with its modified type II properties should behave similarly with respect to requirement of cytochrome P-450 for MEOS activity.

After phenobarbitone treatment, aniline hydroxylase activity and cytochrome P-450 content were increased by 30 and 60 per cent respectively in guinea pigs, but MEOS activity and the rate of ethanol clearance from the blood were unchanged, (tables 9 and 10). As MEOS activity was not induced by phenobarbitone and was not dependent on cytochrome P-450 content, it does not appear to have properties associated with 'true' microsomal enzymes.

In human studies MEOS activity, measured as NADPH dependent ethanol oxidizing system activity, was increased in patients with liver disease (tables 22 and 23), although Schoene et al¹⁴⁹ have shown that cytochrome P-450 content and aminopyrine demethylase activity are reduced in chronic liver disease. From these studies it is again apparent that MEOS activity is not dependent on cytochrome P-450 for activity.

As MEOS does not show changes in activity similar to established microsomal drug metabolizing enzymes, and is not dependent on cytochrome P-450 for activity, it cannot be considered a 'true' microsomal drug metabolizing enzyme. MEOS activity is therefore more likely to be a product of NADPH oxidase and catalase activities, rather than that of a distinct microsomal oxidase⁴⁵.

The increase in the activity of the NADPH dependent ethanol oxidizing system in patients with liver disease may reflect an increase in NADPH oxidase activity, as this is considered the rate limiting enzyme in the above alternative pathway to MEOS³¹. Lieber and De Carli³⁹ have shown that chronic ethanol treatment results in an increase in NADPH oxidase activity in rats, and they suggest that the increased production of H_2O_2 by this increased enzyme may play a role in the pathogenesis of liver damage via enhanced lipid peroxidation. Lundquist¹⁵⁴ has suggested that under pathological conditions an increase in the breakdown products of purine metabolism might (presumably via the xanthine oxidase system) give rise to H_2O_2 formation, although this system has not been quantitated. Glutathione peroxidase, which utilizes most of the H_2O_2 generated outside of the peroxisomes¹⁵⁵, is also reduced in liver disease¹⁵⁶. Under pathological conditions the above systems can all give rise to increased amounts of H_2O_2 , and may contribute to the pathogenesis of liver damage via enhanced lipid peroxidation. The increase in NADPH dependent ethanol oxidizing system activity, in patients with liver disease, may therefore reflect the increase in pathological changes taking place.

In conclusion, when ADH activity is normal, as in the guinea pig experiments, no further enzyme systems appear to be required for ethanol metabolism; although in patients with liver disease, where ADH activity is reduced by 50 per cent, other enzymes may play a quantitatively more important role in ethanol metabolism, as ethanol clearance rates are unchanged in these patients^{146, 147}. It is suggested that the increase in the activity of the NADPH dependent ethanol oxidizing system in patients with liver disease may be an adaptive increase, which compensates for the low ADH activities found in these patients. Normal rates of ethanol metabolism can thus be maintained.

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